University of Alberta

The Influence of Pancreatic Oxygenation during Hypothermic Preservation on Islet Isolation Outcomes

by

Mohammadreza Mirbolooki ©

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Experimental Surgery

Department of Surgery

Edmonton, Alberta
Fall 2008

Library and
Archives Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-46385-7
Our file Notre référence
ISBN: 978-0-494-46385-7

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

University of Alberta

Library Release Form

Name of Author: Mohammadreza Mirbolooki

Title of Thesis: The Influence of Pancreatic Oxygenation during Hypothermic Preservation on Islet Isolation Outcomes

Degree: Doctor of Philosophy

Year this Degree Granted: 2008

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as herein before provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatsoever without the author's prior written permission.

Dr. Mohammadreza Mirbolooki

Abstract

Compelling evidence that perfluorocarbons alone or in combination with UW overcomes the problems associated with pancreas preservation prior to islet isolation is lacking and difficult to obtain. This research is focused on deeper understanding of the underlying cellular pathways related to hypothermic preservation facilitating hypothesis-driven evidence-based development of truly optimized preservation methods.

In this thesis, male Sprague-Dawley rats' pancreas was employed for testing the mechanisms involved in islet death as the whole pancreas is assumed oxygenated. Intraductal injection was not a suitable way to oxygenate the pancreas with the studied oxygen carriers as all groups showed significant lower values than fresh tissue. Dissolved oxygen level in UW solution with continuous bubbling of oxygen was higher as compared to pre-oxygenated TLM. In spite of the huge amount of dissolved oxygen in the PFC layer, it was partially released to the UW solution in TLM group. During 24 hours of preservation, the continuous bubbling of oxygen method significantly improved energy profile of pancreatic tissue as compared to TLM. The improvement was comparable to the fresh values and switched the anaerobic metabolism to aerobic one effectively.

Glucose supplement (5mM) in UW could not improve energy profile more than oxygenation alone and shifted the metabolism to anaerobic pathway. However, it could delay necrosis to 12 hours after preservation as compared to bubbling of oxygen alone. There was no significant lipid peroxidation induced by tissue oxygenation, however, the GSH values decreased significantly with bubbling of oxygen. Glucose supplement could reverse antioxidant capacity loss induced by continuous oxygenation. Continuous bubbling of oxygen prevented apoptosis in a more effective and consistent way during 12 hours of preservations as compared to TLM. Apoptosis observed in pancreatic tissue was mitochondria-dependent, mediated by change in caspase 9 initiated by both hypoxia and hypoglycemia and mitochondria-independent mediated by change in caspase 8 initiated by hypoglycemia. Continuous bubbling of oxygen significantly improved islet yield and function after isolation as compared to TLM. Big changes of DNA fragmentation, small changes in caspases, and increase in mitochondrial function leads to thinking to another possible pathway, so called caspase-independent apoptosis (AIF, and EndoG) which needs to be further studied.

Taken together, oxygenation of pancreas through bubbling plus glucose supplement of UW solution shows superior results in islet yield, function, pancreatic tissue energy profile, apoptosis, necrosis, and intracellular antioxidant capacity as compared to current pancreas preservation method.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my supervisors Dr. Jonathan RT Lakey and Dr. Thomas A Churchill who inspired me to pursue research during the last four years and forever. The completion of this dissertation would not have been possible without their tireless support, invaluable guidance, encouragement, and dedication.

I wish to recognize and sincerely thank the members of my committee during the last four years- Dr. Aziz Ghahary, Dr. AM James Shapiro, Dr. Breay W Paty, Dr. Locksley E McGann, and Dr Ray V Rajotte. It was a privilege to work under the direction of them in both clinical and experimental islet transplantation settings. I am indebted to Dr. Gina Rayat, and Dr. Peter Senior for taking time to critically review my thesis and participating in my candidacy exam. I would also thank Dr. Patrick McDonald who was a great source of motivation, encouragement and advice. I am like wise grateful to my external reader- Dr. Barry J Fuller and external examiner Dr. Karim Fouad for taking time to critically review my thesis.

I wish to acknowledge several individuals, who have facilitated the work for completion of this thesis. Julie Ryan, Rena Pawlick, Deb Dixon, Helena Miszta-Lane, Alana Eshpeter, Dr. Ming Chen, Dr. Linfu Zhu, Greg Olson, Christine Cook, Geneva DeMeyer, Allan Muir, Rosemarie Henley, Colleen Ruptash and Dawne Colwell from Surgical Medical Research Institute and Dr. Tatsuya Kin, Dr. Wendy Zhai, Brad Richer, Shawn Rosichuk, Deborah McGhee-Wilson, Richard Wilson and Doug O'Gorman from Clinical Islet Isolation Laboratory.

I owe special thanks to my friends and colleagues Dr. Payam Salehi, Dr. Mahra Nourbakhsh and Dr. Hossein Arefanian without whom I could never have completed this work. I also recognize that this work would not have been possible without the assistance of many other people whom I have not named.

I am appreciative of the financial support from the Diabetes and Research Wellness Foundation (DRWF), Juvenile Diabetes Research Foundation (JDRF), Faculty of Graduate Studies and Research (FGSR), Graduate Student Association (GSA) and University of Alberta.

Finally, I am deeply indebted to my wife Sheila, my son Ryan, my parents Mohammad and Masoomeh, and my sisters Negar and Narges and my in-laws Abbas, Jila and Shervin for their constant patience, support and encouragement.

This Thesis is Dedicated to All Individuals with Type 1 Diabetes Around the World.

Table of Contents

Chapter Page

One **Pancreas Preservation and Challenges..1**

Diabetes Mellitus and Transplantation of Pancreatic Tissue..............2

Physiology of the Pancreas and the Islet of Langerhans...................4

Pancreas Preservation...6

Euro Collins Solution

Histidine-Tryptophan-Ketoglutarate Solution (Bretschneider)

University of Wisconsin Solution

Los Angeles Preservation Solution-1

Celsior Solution

Two-Layer Method

Hypothermic Perfusion of Pancreas

Metabolic Changes during Pancreas Preservation.........................19

Hypothermia and Hypoxia

Apoptosis

Ischemia Reperfusion Injury

Oxygen Diffusion Limitations...27

Oxygen Solubility and Diffusion: Aqueous versus Perfluorocarbon

Effects of Surface Tension on Oxygen Diffusion

Oxygen Diffusion within Tissue

Oxygen diffusion in pancreas using TLM

Summary...33

General Hypotheses...34

Bibliography..35

Two **Methodology..42**

Rational & Design..43

Animals

Surgical Procedures

Experimental Groups

Partial Oxygen Pressure..45

Tissue Sampling and Time Points 46

ATP, ADP, AMP Assays

Chapter **Page**

Lactate
Malondialdehyde (MDA)
Glutathione (GSH)
DNA Fragmentation
Caspases 3
Caspases (8,9)
Protein Assay
Histology
Immunohistochemistry
Solution's measurements...60
Solution's pH
LDH release
Islet Isolation...61
Islet Yield
Membrane Integrity Fluorescent Staining
Islets Insulin Release Function
Statistics...70
Bibliography...71

Three **Intraductal Oxygenation of Pancreas during Hypothermic Preservation...72**
Introduction 73
Methods 74
Results 75
Discussion 78
Bibliography 80

Four **Continuous Oxygenation of Pancreas during Hypothermic Preservation...82**
Introduction 83
Methods 84
Results 84
Discussion 87

Chapter Page
Bibliography 89

Five Continuous Bubbling of Oxygen during Pancreas Preservation: Effects and Mechanisms...91
Introduction 92
Methods 93
Results 94
Discussion 109
Bibliography 116

Six Glucose Supplement in UW solution: Effects and Mechanisms ..120
Introduction 121
Methods 122
Results 123
Discussion 137
Bibliography 141

Seven Conclusion..144
Dissertation Findings and Implications 145
Future Directions 149
Bibliography 151

List of Tables

Table **Page**

1-1 Components and their concentrations of each preservation Solutions....9

List of illustrations

Figure **Page**

1-1 Confocal micrographs, showing representative immunostained pancreatic sections containing islets of Langerhans from human (*A*) and mouse (*B*) 5

1-2 Bicyclic perfluorocarbon 15

1-3 Oxygen saturation curves for PFC and hemoglobin 15

1-4 ATP turnover of cells as a function of time exposed to anoxia or hypothermia........22

1-5 Intrinsic and extrinsic pathways leading to apoptosis 24

1-6 Mechanism of ischemia reperfusion injury 26

1-7 Oxidative stress in ischemia reperfusion 27

1-8 Oxygen release from PFC: one-layer versus two-layer method 30

1-9 Oxygenated fraction of rat and human pancreas during TLM storage..33

2-1 Rat pancreas procurement 44

2-2 Schematic Islet count under the microscope 64

2-3 Islet Quantification Sheet 65

2-4 Islet viability with the fluorescence microscope 67

3-1 Adenosine tri-phosphate values of pancreatic tissues preserved in HBSS with intra-ductal injection of HBSS, pre-oxygenated solutions of HBSS, PFC, and ePFC........75

3-2 Total adenylate values of pancreatic tissues preserved in HBSS with intra-ductal injection of HBSS, pre-oxygenated solutions of HBSS, PFC, and ePFC........76

3-3 Energy charge values of pancreatic tissues preserved in HBSS with intra-ductal injection of HBSS, pre-oxygenated solutions of HBSS, PFC, and ePFC........77

3-4 ADP-to-ATP ratio values of pancreatic tissues preserved in HBSS with intra-ductal injection of HBSS, pre-oxygenated solutions of HBSS, PFC, and ePFC........77

Figure **Page**

4-1 ATP content of pancreatic tissue during 24 hours of cold preservation in HBSS with & without continuous bubbling of oxygen 85

4-2 Toatal adenylates content of pancreatic tissue during 24 hours of cold preservation in HBSS with & without continuous bubbling of oxygen 86

4-3 Energy charge content of pancreatic tissue during 24 hours of cold preservation in HBSS with & without continuous bubbling of oxygen 86

4-4 ADP/ATP ratio of pancreatic tissue during 24 hours of cold preservation in HBSS with & without continuous bubbling of oxygen 87

5-1 Oxygen partial pressure in UW solution (%) during 24 hours of rat pancreas preservation 95

5-2 ATP level of pancreatic tissue during 24 hours of rat pancreas preservation 96

5-3 Total adenylates level of pancreatic tissue during 24 hours of rat pancreas preservation 97

5-4 Energy charge level of pancreatic tissue during 24 hours of rat pancreas preservation 97

5-5 ADP/ATP ratio level of pancreatic tissue during 24 hours of rat pancreas preservation 98

5-6 Lactate level of pancreatic tissue during 24 hours of rat pancreas preservation 99

5-7 pH level of UW solution during 24 hours of rat pancreas preservation....................100

5-8 GSH level of pancreatic tissue during 24 hours of rat pancreas preservation 101

5-9 MDA level of pancreatic tissue during 24 hours of rat pancreas preservation 101

5-10 DNA fragmentation level of pancreatic tissue during 24 hours of rat pancreas preservation 102

5-11 Caspase 3 level of pancreatic tissue during 24 hours of rat pancreas preservation 103

Figure **Page**

5-12 Caspase 8 level of pancreatic tissue during 24 hours of rat pancreas preservation 104

5-13 Caspase 9 level of pancreatic tissue during 24 hours of rat pancreas preservation 104

5-14 LDH release level of pancreatic tissue during 24 hours of rat pancreas preservation 105

5-15 Hematoxylin and Eosin staining of pancreatic tissue containing an islet after 24 hours of preservation 106

5-16 Insulin staining of pancreatic tissue containing an islet after 24 hours of preservation 106

5-17 Glucagon staining of pancreatic tissue containing an islet after 24 hours of preservation 107

5-18 Post-purification viable islet yield after 24 hours of pancreas preservation in different groups 109

5-19 Stimulation index of isolated islets after 24 hours of pancreas preservation in different groups 109

6-1 Oxygen partial pressure in UW solution (%) during 24 hours of rat pancreas preservation 123

6-2 ATP level of pancreatic tissue during 24 hours of rat pancreas preservation 124

6-3 Total adenylates level of pancreatic tissue during 24 hours of rat pancreas preservation 125

6-4 Energy charge level of pancreatic tissue during 24 hours of rat pancreas preservation 126

6-5 ADP/ATP ratio level of pancreatic tissue during 24 hours of rat pancreas preservation 127

6-6 Lactate level of pancreatic tissue during 24 hours of rat pancreas preservation 127

6-7 pH level of UW solution during 24 hours of rat pancreas preservation 128

6-8 GSH level of pancreatic tissue during 24 hours of rat pancreas preservation 129

Figure **Page**

6-9 MDA level of pancreatic tissue during 24 hours of rat pancreas preservation 129

6-10 DNA fragmentation level of pancreatic tissue during 24 hours of rat pancreas preservation 130

6-11 Caspase 3 level of pancreatic tissue during 24 hours of rat pancreas preservation 131

6-12 Caspase 8 level of pancreatic tissue during 24 hours of rat pancreas preservation 132

6-13 Caspase 9 level of pancreatic tissue during 24 hours of rat pancreas preservation 132

6-14 LDH release level of pancreatic tissue during 24 hours of rat pancreas preservation 133

6-15 Hematoxylin and Eosin staining of pancreatic tissue containing an islet after 24 hours of preservation 134

6-16 Post-purification viable islet yield after 24 hours of pancreas preservation in different groups 135

6-17 Stimulation index of isolated islets after 24 hours of pancreas preservation in different groups......136

List of abbreviations

ATP	Adenosine tri-phosphate
DCCT	Diabetes Control and Complications Trial
EC	Euro Collins
FCS	Fetal calf serum
FFA	Free fatty acid
HBSS	Hanks' balanced salt solution
HTK	Histidine-tryptophan-ketoglutarate
I/R	Ischemia-reperfusion
IDDM	Insulin-dependent diabetes mellitus
IE/kg	Islet Equivalent per kilogram body weight
IU/kg	International unit per kilogram body weight
KH_2PO_4	Potassium hydrogen phosphate
L	Liter
LAP-1	Los Angeles preservation solution
mM/L	Mili-mole per liter
PFC	Perfluorocarbon
rpm	revolutions per minute
SG/EB	SytoGreen and ethidium bromide
SOD	Superoxide dismutase
TCM-199	Tissue culture medium–199
TLM	Two-Layer Method
UW	University of Wisconsin
WHO	World Health Organization
WIT	Warm ischemia time

Chapter One

Pancreas Preservation and Challenges

Diabetes Mellitus and Transplantation of Pancreatic Tissue

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease caused by the progressive destruction of the insulin secreting β-cells of the islets of Langerhans [1]. In this type of diabetes, daily treatment with exogenous insulin is required to assist in the regulation of metabolism to support life. The discovery of insulin, by Banting and Best in 1922, transformed diabetes from a fatal disease to a chronic illness, which has been treatable yet, still is accompanied by severe complications and premature death. The Diabetes Control and Complications Trial (DCCT) of 1993 and its follow-up report in 1997 established that aggressive control of blood glucose lowered (but did not correct) glycated hemoglobin (HbA_{1C}) values and significantly delayed the progression of chronic diabetic complications [2]. The United Kingdom Prospective Diabetes Study Group (UKPDS) of Type 2 diabetics with microvasculature disease reported similar findings [3]. These studies confirmed unequivocally that tight control of blood glucose is essential if the microvascular complications are to be prevented. However, even with aggressive medical management, exogenous insulin therapy is not able to completely reproduce the tight regulatory control of blood glucose levels in diabetic patients that is exhibited in healthy individuals. Moreover, the effects of chronic hyperglycemia and peripheral hyperinsulinemia are believed to accelerate diabetic microangiopathy [4].

It is not surprising then, that surgically replacing the cells/tissue that are defective in diabetic patients is able to reestablish and maintain long-term glucose homeostasis with near-perfect feedback regulatory control [5]. This replacement can take the form of replacing the entire organ (pancreas transplantation) or, with the advent of new technology, replacing only the affected cellular component (by transplantation of ß-cell containing islets). Pancreas transplantation is the standard therapy for insulin-dependent diabetics (Type 1) with established or imminent end-stage renal disease [6]. The procedure is technically demanding and continues to have significant peri-operative mortality and morbidity despite refined surgical techniques, effective immunosuppression modalities, anti-viral prophylaxis and post-transplant monitoring [7,8]. In contrast, islet transplantation with its reduced antigen load, technical simplicity and low morbidity has the potential to dramatically improve the quality of life of individuals with type 1 diabetes.

Islet transplantation involves the extraction of islets of Langerhans from organ donors through a series of complex steps involving physical and enzymatic digestion, followed by purification of the multi-component cellular mixture. Transplantation of the purified islets involves percutaneous, intra-portal injection (under radiographic guidance, Computerized Tomography); the infused islets migrate to the liver where they remain to affect a tighter regulatory control of blood glucose. The surgical procedure itself takes 15-20 min, requires no OR/theatre time and is performed in the radiology ward under local anesthesia with outpatient status. Ultimately, the hope is that transplantation of only the affected tissue/cells will control glucose more stringently and consequently reduce the complications that all diabetics are at risk for as a result of years of poorly regulated systemic glucose levels.

Although progress in finding a cure for diabetes has been slow, the first human islet transplantation occurred in 1989 by the Edmonton Group [9] and was sporadically explored throughout the world during the following years. In 2000, the Edmonton Group published a landmark paper in the New England Journal of Medicine documenting seven diabetic patients (without end stage renal failure) that had normalized blood glucose level following islet transplantation. This study has rekindled considerable interest worldwide in the struggle to find a true cure for type 1 diabetes. Significant advances in pancreas preservation, islet isolation and purification technology, novel immunosuppressants, tolerance strategies, and effective antiviral prophylaxis have found renewed interest and there is a developing collaboration between numerous centres worldwide.

Physiology of the Pancreas and the Islet of Langerhans

The endocrine (hormone-producing) cells of the pancreas are grouped in the islets of Langerhans. Discovered in 1869 by the German pathological anatomist Paul Langerhans, the islets of Langerhans constitute approximately 1 to 2% of the mass of the pancreas. There are about one million islets (~1 g) in a healthy adult human pancreas (60-120 g), which are interspersed evenly throughout the organ. Each islet contains approximately one thousand cells and is 50-500 μm in diameter. Islets are richly vascularized by direct arteriolar blood flow and receive up to 15% of total arterial flow; oxygen tensions has been measured to be twice as high as in islets (40 mmHg) as those in exocrine regions of the pancreas (22 mmHg) [10]. Hence, it is not surprising that islets are sensitive to periods of low oxygen such as those experienced during static organ storage [11].

Islets contain α, β, δ, and pp cells, arranged in a random manner in human islet preparations, as depicted by recent confocal microscopy imaging studies [12] (Figure 1-1A). The paracrine feedback system of the islets of Langerhans has the following structure:

a. Insulin activates beta cells and inhibits alpha cells.
b. Glucagon activates alpha, which inhibits beta cells and delta cells.
c. Somatostatin inhibits alpha cells and beta cells.

It is important to note that the electrical activity of isolated cells from islets is markedly different from an intact islet; hence there is significant interaction between the various cell types within an islet and the close physical proximity and/or direct contact may be required for the appropriate physiological response. There are species-specific differences in the types and distribution of cell types within an islet of Langerhans. Interestingly, mouse islets are primarily composed of insulin-expressing cells clustered within the central core; a much smaller number of glucagon-expressing cells (and somatostatin-expressing cells) are localized to the periphery of the islet (Figure 1-1B). Somatostatin- and PP-expressing cells constitute a minority in islets from all species. It has been observed that β-cells are more sensitive to various apoptotic stimuli such a hypoxia which could be correlated with either hypoxia inducing factor-1 alpha expression in these cells [13] or their core location within an islet. To determine whether the size of islets could influence the success rate of islet transplantations in rats, MacGregor et al. showed that smaller islets were approximately 20% more viable, with large islets containing a scattered pattern of necrotic and apoptotic cells or central core cell death. Small islets in culture consumed twice as much oxygen as large islets and released three times more insulin under basal conditions than did large islets [14].

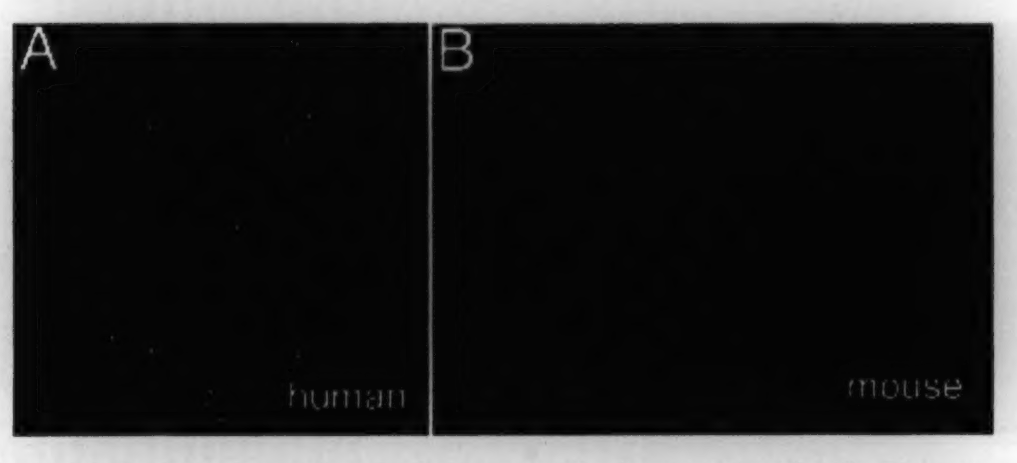


Figure 1-1: Confocal micrographs, showing representative immunostained (Insulin, Glucagon, Somatostatin) pancreatic sections containing islets of Langerhans from human (*A*) and mouse (*B*).[12]

Pancreas Preservation

Pancreas transplantation is the standard therapy for insulin-dependent diabetics with established end-stage renal disease [6]. The surgical procedure itself is technically demanding and continues to have significant peri-operative mortality and morbidity despite refined surgical techniques, effective immunosuppression modalities, anti-viral prophylaxis and post-transplant monitoring [7,8]. In contrast, islet transplantation with its reduced antigen load, technical simplicity and low morbidity has the potential to prevent chronic complications and improves quality of life. The evolution of clinical islet transplantation has made islet allografting a practical treatment for patients with type 1 diabetes. However, offsetting this success is the increasing discrepancy between the availability of donor organs and the demand for transplantable islets. In its present form, less than 0.5% of all affected patients can benefit from islet transplantation [15].

The method of pancreatic preservation employed prior to islet isolation has been purported to be a major limiting factor affecting the quality of islets isolated for transplantation [16]. Currently, the main method of pancreatic preservation relies on the suppression of cellular metabolism by hypothermia. Typically this takes place in a multi-visceral organ procurement, during which the abdominal organs are procured following a common intra-aortic flush with an established preservation solution. The composition of this solution is important so that pancreatic viability is maintained during hypothermic storage and to ensure that effective reperfusion is possible immediately upon implantation. During the last 3 decades, several solutions have been used for hypothermic preservation of the pancreas. These solutions have been designed to address biological and physiological requirements for survival in a low temperature/low oxygen environment. Depending on preservation solution, the basic ionic composition may differ dramatically. Intra-cellular type preservation solutions that are rich in K^+ and low in Na^+ include Bretschneider's (HTK) Solution, Los Angeles preservation solution (LAP-1), Eurocollin's solution and the gold standard, University of Wisconsin (UW) solution. Whereas extra-cellular type solutions that are high Na^+ and low K^+ (eg Celsior) are much fewer in number but have also been utilized for various organs with no apparent differences in preservation efficacy. Some of these solutions have been modified by adding nutrients, antioxidants and anti-apoptotic agents to improve pancreatic viability during extended storage and hence affect superior islet isolation outcomes. Despite marked differences between various preservation solutions and significant differences in constituents, it is generally accepted that the cold ischemic time (storage time) is kept as short as possible. The following sections of this chapter outline the advantages and disadvantages of different preservation solutions and discuss the pathophysiology of the pancreas during extended hypothermic storage and its effect on the quantity and quality of the isolated islets available for transplantation.

Euro Collins solution

In the early 1970s, a hypothermic hyperosmolar solution was introduced as having an ionic composition broadly comparable with intracellular fluid, but without colloid additives (Table 1-1). This solution has been employed to successfully preserve canine kidneys for over 48 hours [17]. The first variants of Collins solutions had high concentrations of potassium, magnesium sulfate, and glucose. In an attempt to increase the osmolality of Collins solution, the glucose concentration was raised (Euro Collin's solution). However, the use of glucose to maintain increased extra-cellular osmolality was potentially problematic for the intra-abdominal organs for two reasons. Firstly, long-term preservation of an organ is associated with increased glucose permeability. The gradual diffusion of glucose into the intracellular spaces diminishes the osmolality of the remaining solution and hence, cannot be relied on to prevent cellular edema [18]. Secondly, glucose is a primary substrate for anaerobic glycolysis, which takes place during hypothermia. Consequently, if glucose is available in large amounts, an over-amplified production of lactate and protons may exacerbate tissue acidosis. Never the less, the EC solution became the first standard solution for cold storage of kidney with reproducible success [19]. Although EC solution was used initially for pancreas preservation, as research progressed, evidence indicated that the preservation of pancreatic tissue could be improved upon with alternative solutions.

In 1990, a study compared the preservation of porcine pancreas with the standard EC solution to the cardioplegic histidine-tryptophan-ketoglutarate (HTK) based Bretschneider's solution. Superior protection was clearly afforded by the HTK solution following as much as 24 h cold storage, by reducing the detrimental effect of proton accumulation (generated from glycolysis), promoting flux through Krebs' cycle (with ketoglutarate, the net result was an increase in arteriovenous flow rate, and superior aerobic metabolism upon reperfusion [20]. Three years later, the same group showed that immediate post-ischemic organ quality and pancreatic function after protection with HTK was similar to preservation with University of Wisconsin (UW) solution; furthermore both were superior to tissue preserved with EC solution [21]. In 2003, preservation of the pancreas with UW solution and EC solution was assessed by X-ray microanalysis and demonstrated that intracellular calcium sequestration in cellular organelles remained intact in tissues preserved with UW solution; conversely, intracellular calcium was poorly controlled when treated with EC solution [22]. Consequently, EC solution is considered inferior to other preservation solutions such as UW and HTK and is no longer employed for clinical pancreas preservation [23]. It should be noted however, that it is used during pancreas cannulation in the process of islet isolation, but not where the pancreas is subjected to a prolonged ischemic period [24].

Table 1-1: Components and their concentrations of each solution. All units are mmol/L unless stated [25].

	UW	HTK	LAP-1	Celsior	EC
Membrane stabilizers					
Lactobionate	100		100	80	
Raffinose	30				
Mannitol		30	30	60	
HES	50g/L				
Tryptophan		2			
Glucose					182
Buffers					
Phosphate	25		15		57.5
Histidine		198		30	
HCo_3^-					10
Energy substrates					
Magnesium	5	4	5	13	
Adenosine	5				
Ketoglutarate		1			
FRIs & ORSSs					
Gluthathione	3			3	
Allopurinol	1				
SOD			$30x10^3$ U/L		
Nicotinamide			5		
Glutamate				20	
Electerolytes					
Na+	30	15	30	100	10
K+	120	10	120	15	115
Calcium		0.015		0.25	
Sulphate	5		5		15
Chloride		28		28	
Hydroxide	100		100		
Osmolality	320	310	320	340	355
pH	7.4	7.4	7.3	7.3	7.4
Additives					
Insulin	100 U/L				
Penicillin					
Dexamethazone	8 mg/L				

HES: Hydroxyethyl starch; FRI: Free radicals inhibitors; ORS: Oxygen reactive species scavangers; SOD: Superoxide dismutase

Histidine-Tryptophan-Ketoglutarate solution (Bretschneider)

The 'HTK' solution, as alluded to in the previous section, was developed in the 1970s by Bretschneider as a cardioplegic solution [26], and is being used increasingly for both kidney [27] and liver [28] transplantation, typically in European centers. HTK contains relatively low potassium and sodium, an effective buffering agent (histidine) and an osmotic agent (mannitol). Tryptophan is added as a 'membrane stabilizer', and ketoglutarate is added as a metabolic substrate (Table 1-1). HTK solution has been used successfully in experimental pancreas transplantation with results comparable to those of UW solution [29]. Use of the HTK solution demonstrated that warm ischemia and cold storage had little impact on graft viability when used for short-term pancreas preservation. Although there has been some concern regarding extended periods of cold storage in the HTK solution, clinical evidence has clearly demonstrated that HTK and UW had similar efficacy with organs <15 h; the only difference was the greater volume of HTK solution used clinically. Despite the greater volume of HTK solution used, the financial costs associated were significantly lower than with UW solution [30]. Further studies have attempted to promote HTK as an effective preservation solution for the pancreas [31], even out to 48 h of cold storage [32]; there are several centres currently employing HTK solution as their routine preservation solution for pancreas, however, most North American transplant programs would consider the UW solution to be the gold standard preservation solution for intra-abdominal organs.

University of Wisconsin (UW) solution

Research efforts by one of the pioneers of organ preservation, Folkert O. Belzer, and his colleague, James H. Southard, resulted in a development of a preservation solution in the late 1980s based on five philosophies [33]. They developed UW solution containing impermeants (raffinose, lactobionate) to minimize hypothermic induced cell swelling, buffer (phosphate) to prevent intracellular acidosis, colloid (hydroxyethyl starch) to prevent the expansion of interstitial space during flush-out period, free radical inhibitors and scavengers (gluthatione, allopurinol) to prevent injury from oxygen free radicals during ischemia and after reperfusion, and energy precursors (Mg^+, adenosine) for energy metabolism during reperfusion period. They also added vasoactive agents and hormones (steroid, insulin) and penicillin as an antibiotic agent (Table 1-1).

In 1990, it was demonstrated that the UW solution is superior to commonly used solutions for storage of the pancreas prior to islet isolation in rodent models and human tissue up to 20 hours after procurement with respect to both the number of islets recovered, and *in vitro* functional integrity of the islets [34]. However, modified variants of the UW solution (typically called 'modified UW solutions') have demonstrated better results in some experimental studies. As an example, a histidine-containing modified UW solution has been reported to be superior to standard UW solution for rat pancreas preservation [35]. This was most likely a result of increased buffering capacity, which has been well-established in a variety of cells/tissues/organs to be a dominant factor affecting viability. Adenosine, insulin, hydroxyethyl starch and dexamethasone, which are components of the UW solution, were not present in this modified solution, suggesting that not all components of the original UW solution are indeed necessary for effective pancreatic preservation. Functional success rates in diabetic rats receiving pancreases that had been preserved in high-histidine (or a high-Na/low K variant) were 100% after 48 h preservation at 4°C. In contrast, the original UW solution resulted in a marked reduction in post-transplant function in this specific study; only a 44% success rate after 48 h preservation was achieved. There have been numerous studies attempting to delineate the critical components of the UW solution, however, most would agree that although the critical components are lactobionate/raffinose, the osmotic impermeants. Although a colloid is not essential for short-term preservation of kidney and liver, for consistently successful 48-hr preservation of the pancreas, hydroxyethyl starch has been shown to be an important component of the UW solution [36]. Interestingly, replacement of hydroxyethylstarch by dextran-40 in UW solution has also shown successful 72-hour preservation of the canine pancreas [37]. Despite the UW solution being originally formulated for preservation of pancreatic tissue, it was quickly adopted by many centers as an excellent solution for the cold preservation of liver and kidney. The introduction of UW solution into clinical practice allowed the safe and extended preservation of the pancreas for up to 30 hrs with reproducible graft function [38]. To date, the UW solution has been the single most important advance in preservation solution technology against which any novel strategy or solution is compared.

Los Angeles preservation solution 1

Kenmochi and his colleagues developed a new cold preservation solution designated Los Angeles preservation solution no. 1 (LAP-1) in 1998 [39]. They believed that the major reasons for failure of islet grafts to maintain normoglycemia in patients with diabetes were insufficient islet number and quality. Therefore, they attempted to develop an isolation method that yielded high quality islets in larger numbers, paying special attention to the vulnerability of islets to oxidative and mechanical stress. The solution consisted of lactobionate K^+ and D-mannitol as impermeants to inhibit cell swelling by maintaining extracellular osmotic pressure (D-mannitol also is known to have scavenging activity for hydroxy radicals to which β cells are highly susceptible) [40]; KH_2PO_4 as a component of the buffering system; superoxide dismutase (SOD) which would provide protection against hydroxy radicals released by leukocytes and other cells contained in the pancreas; and nicotinamide which would enter islet cells that may have been damaged during the isolation process, protect them from further damage, and support their recovery after being transplanted (Table 1-1). Use of a two-step digestion process and LAP-1 cold preservation solution significantly improved the quantity and quality of islets isolated from human pancreas [41]. Since there have been no recent publications to support the data of Kenmochi *et al.*, further research is required to clarify the efficacy of this solution and to determine whether the statistically significant improvements in islet quantity and quality translate to significant clinical outcome measurements.

Celsior solution

Celsior is a recently developed extracellular-type, low-viscosity preservation solution specifically designed for heart transplantation [42] and already used in clinical liver [43] and kidney [44] transplantation. Its composition reflects the putative shortcomings of UW: hydroxyethyl starch was omitted, the potassium concentration was decreased, calcium was included, and the magnesium concentration was increased. Histidine was added to improve buffering capacity and mannitol replaced raffinose. Moreover, the reduced gluthathione present in Celsior is an effective antioxidant approved for clinical use (Table 1-1). Several studies have provided a direct comparison of Celsior and UW solutions in both experimental and clinical models. Two experimental studies investigating pancreatic preservation and transplantation report conflicting results [45,46]; using an autotransplantation model, one study reported that Celsior was an effective alternative to UW for pancreas procurement where another documented increased ischemia-reperfusion injury when Celsior was used as preservation solution.

In a clinical trial, 105 consecutive procurements were randomized to graft preservation with UW (n=53) solution or Celsior (n=52) solution. Mean cold ischemia times were 11.0 ± 2.1 h for UW compared with 10.8 ± 1.8 h for Celsior. Within the range of cold ischemia time reported, it was reported that UW and Celsior solutions have similar safety profiles for pancreas preservation [47].

The different compositions of UW and Celsior solutions might lead to an increased susceptibility of Celsior-preserved grafts to post-reperfusion edema because of the higher chloride and lower lactobionate concentrations and the lack of oncotic agent, hydroxyethyl starch. However, increased free radical scavenging afforded by mannitol/histidine may be beneficial when comparing antioxidant capacities of the two solutions. UW solution contains glutathione, however, it has been found that most glutathione does not remain in the reduced form and oxidizes following several months of storage on the shelf. Nevertheless, UW solution is now considered the gold standard preservation solution for liver, kidney, and pancreas transplantation and any alteration in preservation solution composition or technology must be compared to established clinical parameters.

Two-Layer Method

In the late 1980s, Kuroda et al. developed a two-layer cold storage method (TLM) using perfluorocarbon (PFC) and UW solution for whole-pancreas preservation [48]. PFC is a hyperoxygen carrier designed to release oxygen into the surrounding tissue more effectively (Figure 1-2). PFC differs from hemoglobin preparations in that it is a synthetic compound formed on a liquid hydrocarbon base. In contrast to hemoglobin, oxygen is not chemically bound to the PFC carrier. PFC takes up and releases oxygen following Henry's linear law, on the basis of the partial pressure of the gas, rather than Barcroft's sigmoidal curve described for hemoglobin (Figure 1-3) [49].

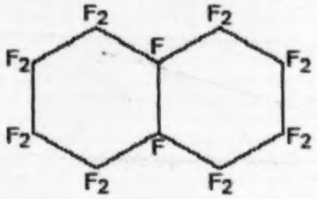


Figure 1-2: Bicyclic perfluorocarbon

Unlike hemoglobin, acidosis, alkalosis, and temperature have little effect on the oxygen delivery of PFC, allowing this compound to be used effectively during cold storage of organs. Since PFC is immiscible with aqueous solutions and has a high specific gravity, in a two layer system with UW solution, the PFC layer remains clear and distinctly separate on the bottom. When incorporating a pancreas, the organ lies on top of the PFC layer with most of the tissue remaining suspended in the aqueous UW solution.

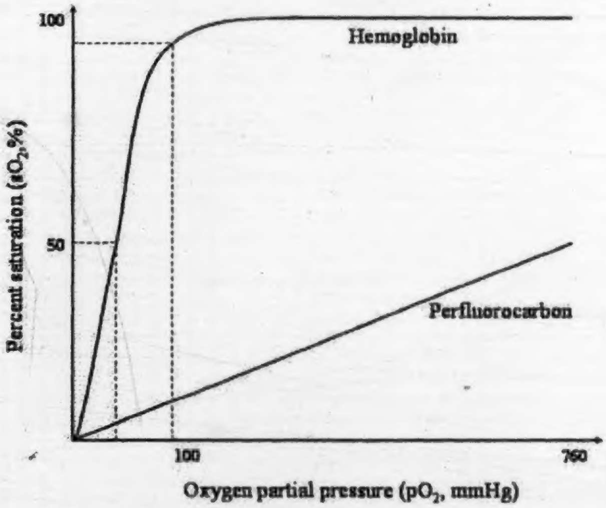


Figure 1-3: Oxygen saturation curves for PFC and hemoglobin [25]

The underlying theory behind use of the two-layer (UW solution–PFC) cold storage method is that due to the high oxygen content of the pre-oxygenated PFC, the pancreas and aqueous UW solution are supplied with oxygen continuously during cold storage, thereby avoiding any hypoxia-mediated injury [50]. Canine pancreases subjected to 90 min of warm ischemia were resuscitated during preservation by the two-layer method at 4°C for 24 to 48 hours [51]. One of the mechanisms of this method is to augment ATP, which maintains cellular integrity and controls ischemic cell swelling. Endogenous substrate for ATP synthesis is not readily available during cold ischemia, however, there is convincing evidence that suggests that ATP is synthesized via direct phosphorylation of adenosine (contained within UW solution) [52]. Since ATP is an essential source of energy for cellular repair, it is not surprising that the ability of a cell/tissue to regenerate or maintain ATP levels within the cell is a key factor in pancreas preservation.

There is a substantial body of evidence outlining the promise of PFC and their use in the TLM (typically with UW solution). Hiraoka et al. achieved insulin independence in type 1 diabetic patients after a single-donor islet transplantation using pancreata subjected to <8h cold storage time with TLM and a novel immunosuppression protocol [53]. Ricordi et al. demonstrated significantly improved islet recovery from older "marginal" donors by using TLM [54]. Matsumoto *et al.*, showed significantly improved islet recovery by instituting a TLM after 6 to 8 hours cold storage in UW solution [55]. Recently, Brandhost *et al.*, presented compelling data that oxygenated perfluorocarbon can be used in a one-layer method (OLM) with comparable outcomes to TLM preservation solution [56]. In Brandhost's study, porcine pancreases were stored for 7 hours with the OLM (PFC alone) or with a TLM (PFC + UW). The authors reported that OLM was significantly superior to TLM in terms of isolation index (calculated as the ratio between IEQ and islet number as an estimate of islet fragmentation) and islet insulin content, however, ATP content, islet yield and graft function remained unaffected. Although the authors suggested that the aqueous layer of the TLM is not essential, there are several confounding factors/issues involving appropriate control groups, PFC oxygen saturation, and core oxygen consumption that may warrant further investigation [57]. A recent paper involving pO_2 measurement using fiber optic sensors in the core of porcine pancreatic tissue preserved with TLM in media saturated with 100% oxygen discovered that at the core of a 1 cm-thick piece of pancreatic tissue, pO_2 values are near-zero when employing the TLM [58]. Accordingly, no beneficial effect of TLM on islet isolation and transplantation outcomes was observed in this study [59].

Hypothermic Perfusion of Pancreas

Hypothermic Perfusion Preservation (HPP) is another technology that has been developed to avoid ischemic injury. Belzer was the creative pioneer who developed one of the first reliable transportable HPP machines [60]. However, for organs other than the kidney, the experimental investigations on HPP did not translate into clinical practice. Florack et al. showed that pancreas preservation by cold storage in high osmolar silica gel filtered plasma is more reliable than pulsatile machine perfusion and provides sufficient time to complete the logistical maneuvers necessary for clinical pancreas transplantation from cadaver donors [61].

Recently, Taylor et al. showed a statistically significant increased islet yield in the hypothermic machine perfusion (HMP) group over static cold storage of pig pancreas in UW after 24 hours of preservation. They concluded that HMP for 24 hours was well tolerated, leading to moderate edema but no loss of function of the harvested islets [62]. Taylor's study was well designed investigating perfusion effects on pancreas preservation in an extended period; however, there is a potential bias as they used KPS in the perfusion group and UW in static group. Leeser et al. did not have this bias but they just perfused human pancreas for 4 hours and concluded that low-flow machine pulsatile perfusion of pancreata with "prolonged" cold ischemia time can result in excellent yield, viability, and function [63].

It has been documented that the provision of oxygen persufflation can improve the viability of rat livers harvested from non-heart-beating donors with 30 to 60 minutes of warm ischemia [64]. Treatment with sufficient oxygen by retrograde persufflation during 24-hour cold storage also could restore life-supporting functions of ischemically damaged kidneys after transplantation [65]. However, oxygen gas persufflation through renal vein caused renal vein thrombosis and required needle perforations on kidney surface allowing gas escape. This method is not feasible in pancreas as the capsule should be kept intact during preservation.

The effects of HPP are multiple and complex, but one fact which was recognized early on in kidneys [66], and has been shown consistently in other organs such as liver or heart, [67,68] is that oxidative energy production via mitochondrial electron transport is sustained at these hypothermic temperatures (around 10 °C). Although the perfusion conditions during HPP are well set for the kidney they are not well established for the liver. HPP was in at the beginning of transplantation, and now has the potential to make a significant renaissance in the era of expanded donor criteria. Renal HPP is an established modality but the philosophical and technical hurdles for transfer to other organs have yet to be overcome [69].

Metabolic Changes During Pancreas Preservation

Although UW solution has proven to be very effective for vascularized pancreas preservation, organ storage in UW solution prior to islet isolation, for even short periods (less than 4 hours), appears to have a profoundly negative impact on islet yield and clinical outcomes. Refined procurement and preservation techniques would allow better allocation of pancreases among islet isolation facilities and organ transplant centers, according to best-matched clinical need, and more efficient use of limited resources.

Hypothermia and Hypoxia

The principle of organ preservation is ingrained in the observation that life processes are temperature dependent chemical reactions, whose sum is the metabolism. Since the 1950s, hypothermia has been known to provide considerable protection against ischemic damage [70]. Kinetic predictions based on thermodynamic considerations (ie. shift in the Maxwell-Boltzmann molecular energy distribution relative to activation energy) indicate that most enzymes of normothermic animals will show a 1.5 to 2-fold decrease in metabolic activity for every 10^0C decrease in temperature [33]. Thus, a decrease in temperature from 37 to 4^0C will decrease the metabolism by approximately 12 fold [71]. Consequently, the preferred and most successful method to date for prolonged organ preservation involves reducing temperature of the tissue. A reduction of 33°C (as in this example) does not halt metabolic processes, but does slow all biochemical events to the point that the tissue will remain viable for at least several hours. This is the time during which surgical teams can prepare for transplant, tissue typing can more effectively match donor: recipient, transport of the organ across country is possible thereby effectively increasing the donor pool.

It has been generally accepted that the most effective temperature of tissue/organ storage is as close to the freezing point of water as possible without the deleterious formation of ice crystals in the tissue [72]. As well, it has been convenient to store a procured organ in a pot of cold preservation solution (pre-cooled in a refrigerator) on ice. Generally, the standard temperature reported for organ preservation has been the temperature of a well-regulated refrigerator, 4°C, regardless of whether the tissues are actually at 4°C or not. There have been a few studies that specifically address the subject of the optimal temperature for intra-abdominal organ preservation. Belzer et al. showed that the viability of cultured kidney tubule cells was greatest at 6^0C [73]. Creatinine clearance post-transplant was significantly improved if the rabbit's kidney was perfusion-preserved at 8^0C rather than at 0^0C [74]. Continuous liver perfusion at 10^0C for 24 h showed significantly higher tissue adenine nucleotide levels than those following continuous perfusion at 4^0C [75]. With respect to pancreatic preservation temperatures, both islet yield and function obtained from pancreases stored at 7-10^0C was superior to those obtained from pancreases stored at 4^0C [76]. However, Inui et al. found that –0.6^0C was superior to 4^0C for islet preservation; functional tests involving both a static glucose challenge and the stimulation index were significantly higher in islets stored at –0.6^0C compared to 4^0C [77]. Despite, the varying evidence throughout the literature, it is likely that 4°C is not necessarily the optimal storage temperature for all tissues/organs, nevertheless, a temperature of 0-4^0C is often employed in current experiments and the clinical practice of placing an isolated organ in a pot of pre-cooled preservation solution on ice in an insulated cooler has not changed.

Under natural physiological conditions, cells are immersed within an extracellular solution high in sodium and low in potassium. This gradient is maintained by the Na^+ -K^+ ATPase pump, which uses adenosine tri-phosphate (ATP) derived from oxidative phosphorylation in the mitochondria. Exposure to hypothermia reduces the activity of the membrane ion exchange mechanisms, specifically the Na^+-K^+ ATPase system which consumes one third of the total cellular energy is preferentially inhibited at lower temperatures [78]. Any reduction in temperature leads to an intracellular accumulation of sodium which in turn leads to an osmotic increase in cell water content, that has been previously termed "cold swelling" [79]. Cold-induced acceleration of Na-H exchange is an additional route for sodium influx [80]. If cold-induced accumulation of sodium remains unchecked, the rise in cytosolic sodium will ultimately lead to membrane depolarization, opening of voltage-dependent Ca^{2+} channels, rapid influx of calcium and initiation of membrane phospholipid hydrolysis (Figure 1-4) [81]. Once initiated, the pathological series of effects leading to necrotic cell death during hypothermia is largely uncontrollable and analogous to irreversible membrane injury. To solve this problem, large impermeant molecules may be added to the preservation solution to match osmotic pressure within the intracellular compartment and counteract the tendency of the cell to take up water and hence, swell. Impermeants that have been used in preservation solutions include: saccharides such as raffinose, lactobionate, manitol, glucose or anions such as phosphate, sulfate, citrate and gluconate. Although, the use of colloids and impermeants represents a major function of an effective preservation solution, ischemia (hypoxia) still remains a limiting factor affecting all statically stored organs/tissues.

Hypoxia exists when oxygen delivery does not meet the demands of the tissue. During pancreas procurement, blood supply and hence oxygen is necessarily interrupted. Therefore, cells cannot continue to meet the energy demands of active ion-transporting systems leading to similar consequences to hypothermia (Figure 1-4) [82]. In the early stages of ischemia (stoppage of blood flow), cellular ATP demands tend to remain constant. This leads to an energetic deficit that can only be made up for by activation of anaerobic ATP supply pathways, namely glycolysis. Anaerobic ATP production cannot sustain the pre-existing energy demands because of the rapid depletion of fermentable substrate available and end products (e.g. lactate and H^+) cannot accumulate indefinitely. Moreover, accumulation of lactic acid due to anaerobic glycolysis results in tissue acidosis which is deleterious to normal cell function. During ischemia, in addition to the usual pathway for lactic acid production, lactate dehydrogenase (LDH) converts pyruvate to lactic acid. The high concentrations of lactic acid not only injure cells directly, but can also activate macrophages, leading to cytokine production and the initiation of an amplified inflammatory response [83]. To prevent acidosis, hydrogen ion buffers can be used in preservation solutions, these include potassium phosphate, sodium bicarbonate, magnesium sulfate and perhaps the most effective buffer of those currently used, histidine.

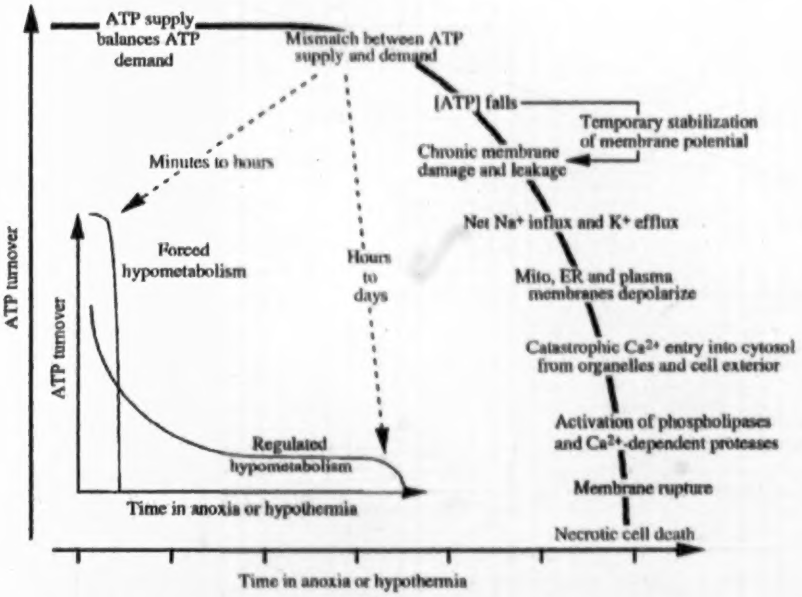

ATP supply balances ATP demand
Mismatch between ATP supply and demand
[ATP] falls
Temporary stabilization of membrane potential
Chronic membrane damage and leakage
Minutes to hours
Net Na+ influx and K+ efflux
Forced hypometabolism
Hours to days
Mito, ER and plasma membranes depolarize
Catastrophic Ca2+ entry into cytosol from organelles and cell exterior
Activation of phospholipases and Ca2+-dependent proteases
Regulated hypometabolism
Membrane rupture
Time in anoxia or hypothermia
Necrotic cell death
ATP turnover
ATP turnover
Time in anoxia or hypothermia

Figure 1-4: ATP turnover of cells as a function of time exposed to anoxia or hypothermia. Mito, mitochondria; ER, endoplasmic reticulum [84].

Apoptosis

As the time of organ storage progresses, the imbalance between ATP supply and demand will eventually result in cell/tissue necrosis (Figure 1-4) or apoptosis (either via extrinsic or intrinsic pathways; (Figure 1-5) [85]. Although research has attempted to reduce necrosis by several aforementioned preservation solution strategies (eg. by adding osmotic impermeants and/or effective buffering agents to the preservation solution), attempts at reducing the controlled cell death of apoptosis have been more difficult to employ. Nevertheless, the basic strategy employed in this thesis, that is 'the provision of oxygen', may be the simplest and easiest method by which apoptosis may be abrogated during pancreatic storage. There are two basic pathways of apoptosis, the intrinsic and the extrinsic pathways (Figure 1-5). The intrinsic or mitochondrial pathway is used extensively in response to extracellular cues and internal insults, but is initiated at the level of the mitochondria. Activation of pro-apoptotic members, such as the Bcl-2 family [86] can occur through proteolysis, dephosphorylation and several other mechanisms [87]. Pro- and anti-apoptotic Bcl-2 family members compete at the surface of the mitochondria, to regulate the exit of cytochrome c from the organelle. Pro-apoptotic signals result in the release of an array of molecules from the mitochondrial compartment. Principal among these is cytochrome *c*, which associates with Apaf-1 and procaspase-9 in sequence (and possibly other proteins) to form the "apoptosome".

The extrinsic pathway signal is initiated at cell surface receptors (death receptor) and typically responds to circulating cytokines. The death-receptor pathway is triggered by members of the death-receptor superfamily. Binding of CD95 ligand to CD95 induces receptor clustering and formation of a death-inducing signalling complex. This complex recruits, via an adaptor molecule FADD (Fas-associated death domain protein), multiple procaspase-8 molecules, resulting in caspase-8 activation through induced proximity [88].

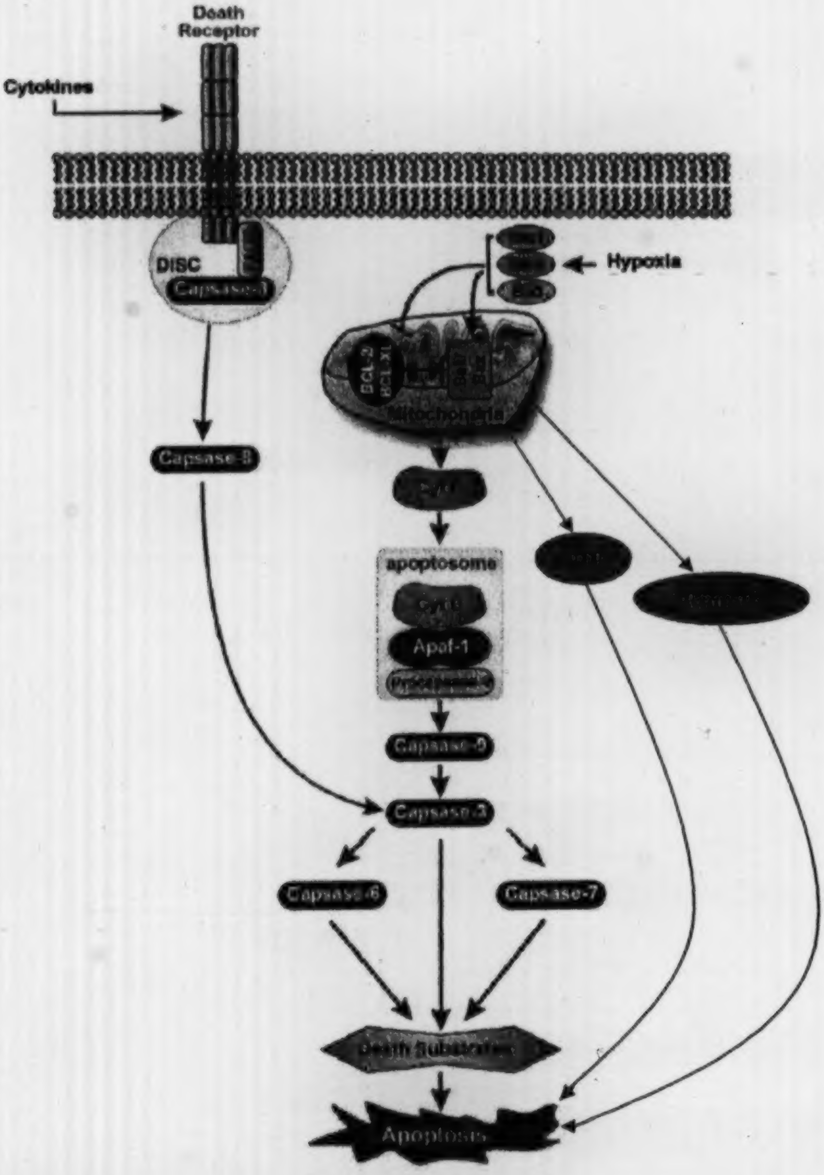

Death Receptor
Cytokines
DISC
Capsase-8
Hypoxia
BCL-2
BCL-XL
Mitochondria
Capsase-8
apoptosome
Apaf-1
Capsase-9
Capsase-3
Capsase-6
Capsase-7
Death Substrates
Apoptosis

Figure 1-5: Intrinsic and extrinsic caspase-dependent pathways [89] and AIF, EndoG mediated caspase-independent pathways leading to apoptosis.

The death-receptor and mitochondrial pathways converge at the level of caspase-3 activation. Caspase-3 activation and activity is antagonized by the IAP proteins, which themselves are antagonized by the Smac/DIABLO protein released from mitochondria. Downstream of caspase-3, the apoptotic programme branches into a series of cellular signals, the sum of which results in the ordered dismantling and removal of the cell and its contents. As complex as the intrinsic and extrinsic pathways are clearly becoming, there are additional death-inducing pathways that are independent of intrinsic/extrinsic mechanisms, since developmental apoptosis remains intact and active in mice that are defective in both caspase-8 and caspase-9 pathways [90].

X-linked inhibitor of apoptosis protein (XIAP) is a potent endogenous inhibitor of apoptosis that is capable of blocking the activation of multiple downstream caspases. Human islets transduced with a XIAP-expressing recombinant adenovirus shown to be resistant to apoptosis and functionally recovered following in vitro stresses of hypoxia and reperfusion injury [91]. Ruthenium Red (a glycoprotein stain that binds to Ca^{2+} binding sites of the mitochondrial Ca^{+2} uniporter) inhibits hypothermic storage-induced Bax translocation from cytosol to mitochondria [92]. Trimetazidine (TMZ, an anti-anginal medication) has shown to be able reduce the inflammatory response prevalent in ischemic kidney injury and rate of apoptosis expression [93]. Although the activation of caspase is most likely a predominant mechanism inducing apoptosis, apoptosis could be mediated by mechanisms that do not involve caspases that need to get clarified. This pathway, termed caspase-independent apoptosis, is characterized by a large scale DNA fragmentation with an early chromatin condensation pattern. Caspase-independent factors, such as apoptosis inducing factor (AIF), endonuclease G (Endo G) and High temperature requirement protein A2 (HtrA2/Omi), might be involved in inducing apoptosis without mediation of caspases in pancreas hypothermic preservation.

Ischemia Reperfusion Injury

Following a period of low oxygen (hypoxia or ischemia), when blood flow and the supply of oxygen is reestablished, damage to the cell/tissue can be exacerbated by a rapid production of harmful oxygen free radicals. Although there is a basal generation of oxygen free radicals via leakage of electrons from mitochondrial respiration (up to 1-3 % of oxygen metabolized is converted to radical formation by this mechanism), a primary source of free radicals that is of particular relevance to organ storage and the decline of high energy adenylates is the hypoxanthine-xanthine oxidase reaction (Figure 1-6). These radicals can cross-link membrane proteins, cleave peptide bonds, alter the function of glycosaminoglycans, and promote DNA disruption (Figure 1-7). The effects of a burst of oxygen radical production upon reoxygenation are often amplified as a result of the depletion of endogenous antioxidant mechanisms (ie. glutathione) that may occur during organ storage.

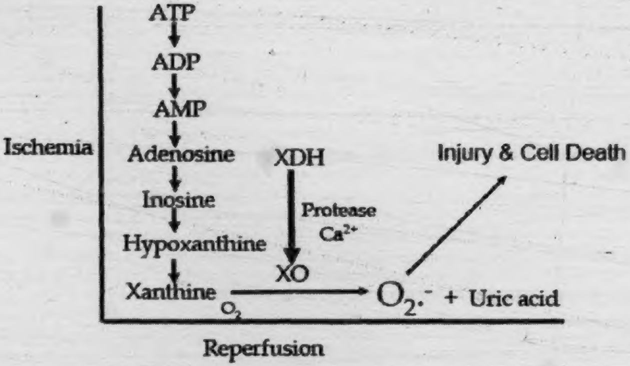


Figure 1.6: Mechanism of ischemia reperfusion injury

Administering exogenous antioxidants like glutathione has long been known to play an important role in protecting the ischemic tissue from reperfusion injury. Several preservation fluids include glutathione as a specific additive to limit oxygen free radical injury [47]. Reduced glutathione combines with reactive oxygen species and free radicals by either an enzyme-catalyzed or via direct chemical reduction to minimize oxidative injury to tissues. Other antioxidants and free radical scavengers used in various preservation fluids include superoxide dismutase, allopurinol, prostaglandin synthesis inhibitors, and vitamin E (a lipid soluble antioxidant).

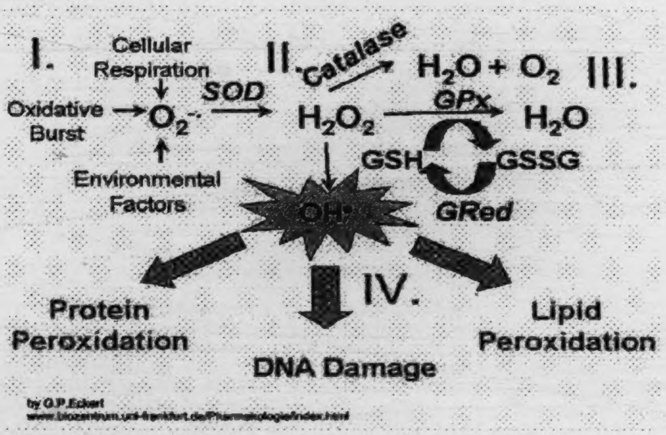

I.
Cellular Respiration
Oxidative Burst
$O_2^{\cdot-}$
SOD
Environmental Factors
II.
Catalase
$H_2O + O_2$
III.
H_2O_2
GPx
H_2O
GSH
GSSG
GRed
OH·
IV.
Protein Peroxidation
DNA Damage
Lipid Peroxidation
by O.P.Eckert
www.biozentrum.uni-frankfurt.de/Pharmakologie/index.html

Figure 1-7: Oxidative stress in ischemia reperfusion [94]

Oxygen Diffusion Limitations

Energy levels within the tissue are soon depleted during ischemic storage because many energy consuming processes are still in operation even at hypothermia [95]. This phase of cold ischemia is, at present unavoidable and results in the gradual deterioration of graft function. Although the effect of two-layer method on canine islet preservation has been well documented to improve islet cell yield and function, few data are available concerning the benefit of TLM in human islet isolation. There has been some published data on human islet isolation that suggests that the two-layer method with UW solution and perfluorodecalin (a common perfluorocarbon with excellent oxygen solubility characteristics) does improve the function of isolated islets following transplantation [96]. However, in a recent clinical study, researchers at the University of Alberta determined that the mass of transplanted islets and functional viability of islet isolated from pancreases preserved with a TLM were no different than islets isolated from organs preserved in UW solution. Patients receiving islets from either TLM or UW-preserved pancreas exhibited positive clinical outcomes (decrease in exogenous insulin requirement) following transplantation. However, there was no significant difference between the two preservation strategies; storage in UW solution was as effective as the TLM. This clinical study brings into question the true merit of routine use of TLM prior to islet isolation.

Oxygen Solubility and Diffusion: Aqueous versus Perfluorocarbon

Perfluorocarbons can dissolve far greater quantities of oxygen than other liquids at comparable pressure [97]. At 1 atm pressure and 25°C temperature, they dissolve 14-fold as much oxygen as aqueous solutions (49 mL O_2 per 100 mL of PFC vs. 3.4 mL O_2 per 100 mL of aqueous solutions: like UW solution) [98]. Discussions of the possible value of PFCs as an oxygen provider during pancreas preservation always refer to their enormous gas-carrying capacity. However, solubility is only a measure of solute uptake and does not distinguish between the solute molecules freely available in the solvent and those bound to it; therefore it is not a parameter that defines the gas transfer capacity of a liquid [99]. Both diffusion and solubility of gases in liquids are correlated with the boiling points, densities, molecular weights and surface tensions of the liquids. Hence, it is vital to our understanding that the diffusion coefficient of gases is equally important, if not more so, than oxygen solubility characteristics when investigating oxygen delivery in preservation solutions/strategies. Numerous measurements of the diffusion coefficient of oxygen through water have been summarized [100] indicating that the diffusion coefficient at 25°C is 2.12×10^{-5} cm^2sec^{-1}. Surprisingly, the diffusion coefficient of oxygen through PFC at 22 °C is reported as 2.0×10^{-5} cm^2sec^{-1}; which is similar to that of aqueous solutions [99]. As a general rule, with a decrease in temperature, oxygen solubility increases, while diffusion coefficients decrease. Hence, although oxygen solubilty of PFC is higher than UW, oxygen diffusitivity in both UW and PFC is similar and may become a limiting factor at the near-zero temperatures employed during organ storage.

Effects of Surface Tension on Oxygen Diffusion

One of the most important factors limiting the diffusion of a gas within a liquid or between liquids is the surface tension of liquid molecules. Surface tension is an effect within the surface layer of a liquid that causes that layer to behave as an elastic sheet. The phenomenon of 'surface tension' is the result of an attraction between the molecules of the liquid by various intermolecular forces. Surface tension is dependent on temperature. For that reason, when a value is given for the surface tension of an interface, temperature must be explicitly stated. The general trend is that surface tension increase with the decreases of temperature. Brandhorst et al. reported that omitting the University of Wisconsin (UW)-layer accelerates oxygen release into the ambient atmosphere by measuring oxygen tensions in PFC pre-charged for One-Layer-Method as compared to that for TLM (Figure 1-8) [101]. Partial oxygen tension was determined sequentially in 500 mL of PFC stored for 30 hours at 4°C in a closed 1000 mL-wide mouth jar. PFC was pre-charged with 100% oxygen for 30 min at a rate of 2000 mL/min was stored alone (OLM, open circles) or covered with 300 mL of UW solution (TLM, filled squares). From this data, it is clear that the one layer method with PFC was able to more effectively liberate dissolved oxygen from PFC without the second 'interfering' aqueous layer. The explanation of this phenomenon lies in the high surface tension of the UW solution (74.9 erg cm^{-2} at 5 °C) compared to that of air.

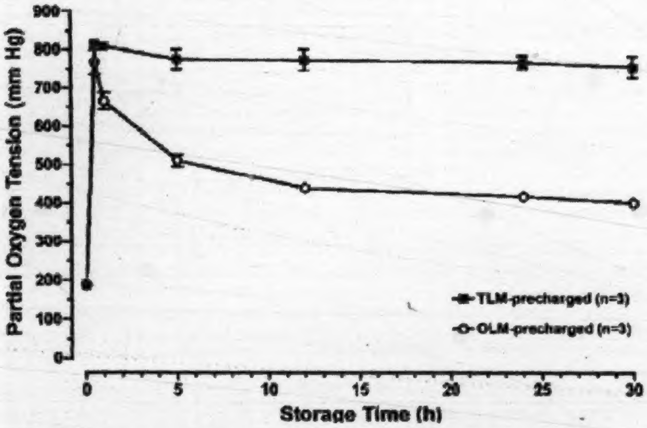


Figure 1-8: Oxygen release from PFC: one-layer versus two-layer method [101].

Oxygen Diffusion within Tissue

There are results in the literature which have shown remarkably low rate of oxygen diffusion through connective tissue in which respiring cells are either infrequent or absent [102]. However, it may seem paradoxical that measurements made for the diffusion coefficient of oxygen through tissues consisting of respiring cells are significantly higher than values calculated for diffusion through an equivalent volume of water at the same temperature. The diffusive flux of oxygen through actively respiring tissues is dependent on two interactive components, diffusion through the aqueous phase of the tissue and diffusion through the membrane component. This may help to explain the somewhat contrasting results for the diffusion of oxygen through respiring liver (1.1×10^{-4} cm^2sec^{-1}) at 37 °C, while diffusion through rat diaphragm (which has a large connective tissue component) was ~1/10 the value at 1.4×10^{-5} cm^2sec^{-1} [103].

The solubility of oxygen through the cell membrane can greatly augment the speed of transport for oxygen by rapidly generating oxygen-rich regions in outer lipid component of the cells throughout a tissue. It has been shown [104] that oxygen partitions preferentially into erythrocyte membranes rather than the surrounding aqueous media, implying that a significant proportion of oxygen movement is facilitated via the hydrophobic domain of the membrane lipid [102]. Gold [105] and Longmuir [106] have independently proposed that there may be a carrier system for oxygen. However, only myoglobin has been found as a soluble readily extractable carrier, and then only in certain types of muscle cells. This has prompted the suggestion that any oxygen carrier must be fixed and immobile as a component of the membrane systems within cells. This idea has led to the realization that it may be the matrix of the membrane itself which might function as a way for the directed and efficient flux of oxygen across tissues. It has been hypothesized that the preferential accumulation of oxygen in the cell membranes may represent a regulatory control mechanism to diminish the risk of 'unscheduled' oxidations and to effectively control flux through glycolysis or through aerobic pathways [107].

Temperature affects oxygen solubility and diffusion in tissue; however the effects are not completely clear and remain somewhat controversial. Bentley et al. [108] investigated the effect of temperature on the oxygen diffusion coefficient (DO_2) of hamster retractor muscle from 11 to 37°C. DO_2 was measured using a non-steady-state technique. DO_2 was 0.84×10^{-5} cm^2sec^{-1} at 11°C and rose exponentially to 2.41×10^{-5} cm^2sec^{-1} at 37°C, producing a temperature coefficient for DO_2 of 4.6%/°C. This value of temperature coefficient is much higher than the previously reported value of 2.5%/°C [109]. Desauliners et al. [110] measured the diffusion coefficient and solubility of oxygen in muscle from striped bass (Morone Saxatilis). Under isothermal conditions, solubility was 3.6×10^{-2} and 6.6×10^{-2} $mlO_2cm\text{-}^3atm^{-1}$ in tissues at 25°C and 5°C, respectively. Because O_2 is more soluble in lipid than aqueous phase, higher solubility in tissues from cold-acclimated animals could be accounted for by the 13-fold increase in lipid content that is known to occur in oxidative muscle of striped bass during acclimation from 25°C to 5°C. Surprisingly, when measured at the two temperatures in acclimated animals, there was no difference in DO_2 values 2.50×10^{-5} and 2.57×10^{-5} cm^2s^{-1}, respectively.

Oxygen Diffusion in Pancreas using TLM

The PFC solution is thought to improve pancreas oxygenation, which is supported by the reported increased ATP concentrations. To my knowledge, though, there is only a single report of direct oxygen partial pressure (pO_2) measurements in a preserved pancreas [111]. In their study, pO_2 measured in canine pancreata at a distance of 5 mm from the surface of the organ was found to be stable at about 15 mm Hg during 5 hours of preservation at 20°C with the TLM. Based on theoretical calculations, the oxygenated rim is less than 1 mm thick at 4 8°C; beyond this rim, pO_2 profiles are virtually zero [112]. For this reason a homogenous diffusion model has been used to describe oxygen [illegible]netration into viable organs/tissue. Schematic sections of cylindrical preserved pancreata with diameter 1 and 2.5 cm showing the predicted oxygenated rim and anoxic core. The predicted oxygenated volume fraction of a preserved pancreas as a function of temperature demonstrates that the diffusion of oxygen has a greater effect on the overall oxygenation state in a smaller piece of tissue. That is to say, smaller pancreases (like rodents or murine organs) will exhibit considerably greater improvements in overall organ oxygenation than in pancreases from larger animals or humans.

Avgoustiniatos et al. predicted the oxygenated volume fraction of rat and human pancreases during preservation with TLM at 4°C (Figure 1-9). This fraction is 100% for the 1- to 2-mm thick head region of a rat pancreas, close to 60% for the 4-mm thick splenic region of a rat pancreas, and less than 20% for a cylindrical 25-mm diameter pancreas [113]. Clearly, these models explain some of the discrepancies between the remarkable effects of TLM when used in small animal experiments compared to clinical studies that demonstrate limited benefit. It has been theorized that if the inert and lipophobic characteristics of oxygen carriers could be substantially reduced, enhanced oxygen-carrier diffusion and oxygen release into the deeper tissue layers may be able to increase islet isolation outcomes in the clinic [113].

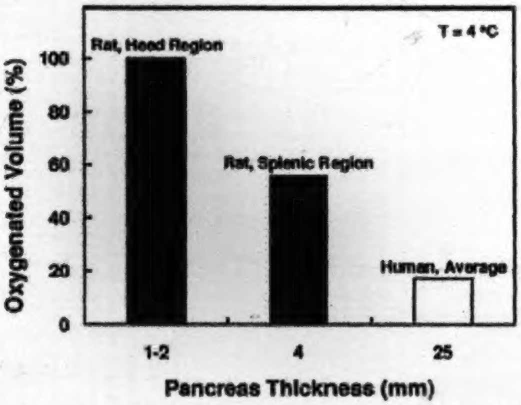


Figure 1-9: Oxygenated fraction of rat and human pancreas during TLM storage [113].

Summary

One of the most important obstacles resulting in the loss of islet cell mass and functional quality is hypoxia-induced necrosis and apoptosis during hypothermic pancreatic preservation. Perfluorochemical-based preservation techniques such as the TLM has shown some promise as the only method that is able to effectively supply oxygen to pancreatic tissue during cold storage [114]. Compelling evidence that PFC alone or in combination with UW overcomes the problems associated with organ preservation is lacking and difficult to obtain for human pancreas. Discrepancies in TLM results may be related to the differences in donor characteristics, organ procurement and lots of other confounding factors. Moreover, although oxygen solubility of PFC is significantly higher than UW, oxygen diffusivity of both UW and PFC are identical. Furthermore, surface tension of UW solution is one of the most important factors limiting the diffusion of oxygen between PFC and an aqueous-based tissue such as the pancreas. The effect of TLM has not been evaluated in a well controlled study yet and the probable mechanisms of resuscitation of ischemically damaged pancreata after cold storage have not been investigated properly as well. This research is focusing on gaining a deeper understanding of the governing principles of oxygen delivery and the underlying cellular processes relating to hypothermic organ preservation that are currently limiting islet isolation outcomes And tries to answer the following questions:

1- Is there any other method that oxygenates the pancreas better than TLM?
2- What are the mechanisms involved in hypoxia induced islets death during hypothermic preservation of pancreas?
3- What is the role of nutrients like glucose in islets death during hypothermic preservation of pancreas?

To answer the above questions, the best model is a pancreas that could be oxygenated as much as possible to limit the confounding factors. Based on Figure 1-9, the best model for this purpose is rat pancreas.

General Hypotheses:

- Intraductal oxygenation of pancreas during hypothermic organ preservation improves pancreatic tissue energy profile
- Continuous bubbling of oxygen during hypothermic organ preservation improves pancreatic tissue energy profile
- Continuous bubbling of oxygen during hypothermic organ preservation prevents apoptosis in pancreatic tissue
- Glucose supplement improves pancreatic tissue antioxidant capacity during continuous oxygenation of hypothermic preserved pancreas

Bibliography

---

[1] Gepts W. Islet morphology in type I diabetes. Behring Inst Mitt. 1984 Jul;(75):39-41.

[2] The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med. 1993 Sep 30;329(14):977-86.

[3] Turner RC, Holman RR. The UK Prospective Diabetes Study. Ann Med. 1996 Oct;28(5):439-44.

[4] Despres JP, Lamarche B, Mauriège P, Cantin B, Dagenais GR, Moorjani S, Lupien PJ. Hyperinsulin-emia as an independent risk factor for ischemic heart disease. N Engl J Med. 1996 Apr 11;334(15):952-7.

[5] Ryan EA. Pancreas transplants: for whom? Lancet. 1998 Apr 11;351(9109):1072-3.

[6] Robertson RP, Davis C, Larsen J, Stratta R, Sutherland DE; American Diabetes Association. Pancreas transplantation for patients with type 1 diabetes. Diabetes Care. 2003 Jan;26 Suppl 1:S120.

[7] Sutherland DE, Gruessner RW, Gruessner AC. Pancreas transplantation for treatment of diabetes mellitus. World J Surg. 2001 Apr;25(4):487-96.

[8] Sutherland DE, Gruessner RW, Dunn DL, Matas AJ, Humar A, Kandaswamy R, Mauer SM, Kennedy WR, Goetz FC, Robertson RP, Gruessner AC, Najarian JS. Lessons learned from more than 1,000 pancreas transplants at a single institution. Ann Surg. 2001 Apr;233(4):463-501.

[9] Warnock GL, Kneteman NM, Ryan E, Seelis RE, Rabinovitch A, Rajotte RV. Normoglycaemia after transplantation of freshly isolated and cryopreserved pancreatic islets in type 1 (insulin-dependent) diabetes mellitus.Diabetologia. 1991 Jan;34(1):55-8.

[10] Carlsson PO, Liss P, Andersson A, Jansson L. Measurements of oxygen tension in native and transplanted rat pancreatic islets. Diabetes. 1998 Jul;47(7):1027-32.

[11] Sweet IR, Khalil G, Wallen AR, Steedman M, Schenkman KA, Reems JA, Kahn SE, Callis JB. Continuous measurement of oxygen consumption by pancreatic islets. Diabetes Technol Ther. 2002;4(5):661-72

[12] Cabrera O, Berman DM, Kenyon NS, Ricordi C, Berggren PO, Caicedo A. The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. Proc Natl Acad Sci U S A. 2006 Feb 14;103(7):2334-9.

[13] Moritz W, Meier F, Stroka DM, Giuliani M, Kugelmeier P, Nett PC, Lehmann R, Candinas D, Gassmann M, Weber M. Apoptosis in hypoxic human pancreatic islets correlates with HIF-1alpha expression. FASEB J. 2002 May;16(7):745-7.

[14] MacGregor RR, Williams SJ, Tong PY, Kover K, Moore WV, Stehno-Bittel L. Small rat islets are superior to large islets in in vitro function and in transplantation outcomes. Am J Physiol Endocrinol Metab. 2006 May;290(5):E771-9.

[15] Mirbolooki M, Shapiro AMJ, Lakey JRT. A perspective on clinical islet transplantation: past, present and developments for future. Immun, Endoc. & Metab. Agents in Med. Chem. 2006; 6(2): 191-208.

[16] Salehi P, Mirbolooki M, Kin T, Tsujimura T, Shapiro AM, Churchill TA, Lakey JR. Ameliorating injury during preservation and isolation of human islets using the two-layer method with perfluorocarbon and UW solution. Cell Transplant. 2006;15(2):187-94.

[17] Liu WP, Humphries AL Jr, Russell R, Stoddard LD, Moretz WH. 48-hour storage of canine kidneys after brief perfusion with Collins' solution. Ann Surg. 1971; 173(5):748-57.

[18] Grino JM, Alsina J, Castelao AM, Sabate I, Mestre M, Gil-Vernet S, Andres E, Sabater R. Low-dose cyclosporine, anti-lymphocyte globulin, and steroids in first cadaveric renal transplantation. Transplant Proc. 1988; 20(Suppl 6):18-20.

[19] Southard JH, Belzer FO. Organ preservation. Annu Rev Med. 1995;46:235-47.

[20] Leonhardt U, Barthel M, Tytko A, Droge M, Siegel EG, Nebendahl K, Kohler H, Creutzfeldt W. Preservation of the porcine pancreas with HTK and Euro-Collins solution: studies in a reperfusion system. Eur J Clin Invest. 1990; 20(5): 536-9.

[21] Leonhardt U, Tytko A, Exner B, Barthel M, Stockmann F, Kohler H, Siegel EG, Nebendahl K, Creutzfeldt W. The effect of different solutions for organ preservation on immediate postischemic pancreatic function in vitro. Transplantation. 1993; 55(1):11-4.

[22] Kozlova I, Roomans GM. Preservation of pancreas tissue during cold storage assessed by X-ray microanalysis. Am J Transplant. 2003; 3(6): 697-707.

[23] Sutherland DE, Gruessner AC, Gruessner RW. Pancreas transplantation: a review. Transplant Proc. 1998 Aug;30(5):1940-3.

[24] Lakey JR, Kobayashi N, Shapiro AMJ, Ricordi C, Okitsu T. Current human islet isolation protocol. Medical Review Co. Ltd: Tokyo, 2004.

[25] Mirbolooki M, Lakey JRT. Pancreas preservation for islet isolation.In: Islet Transplantation and Beta Cell Replacement Therapy, Shapiro AMJ, Shawn AMJ (Eds.) Informa healthcare, 2007, pp: 99-114

[26] Bretschneider HJ. Myocardial protection. Thorac Cardiovasc Surg. 1980; 28(5):295-302.

[27] de Boer J, De Meester J, Smits JM, Groenewoud AF, Bok A, van der Velde O, Doxiadis II, Persijn GG. Eurotransplant randomized multicenter kidney graft preservation study comparing HTK with UW and Euro-Collins. Transpl Int. 1999;12(6):447-53.

[28] Hatano E, Kiuchi T, Tanaka A, Shinohara H, Kitai T, Satoh S, Inomoto T, Egawa H, Uemoto S, Inomata Y, Lang H, Oldhafer KJ, Ringe B, Pichlmayr R, Tanaka K, Yamaoka Y. Hepatic preservation with histidine-tryptophan-ketoglutarate solution in living-related and cadaveric liver transplantation. Clin Sci (Lond). 1997; 93(1):81-8.

[29] Troisi R, Meester D, Regaert B, Jacobs B, Van den Broucke C, Cuvelier C, de Hemptinne B, Hesse UJ. Physiologic and metabolic results of pancreatic cold storage with Histidine-Tryptophan-Ketoglutarate-HTK solution (Custodiol) in the porcine autotransplantation model. Transpl Int. 2000;13(2):98-105

[30] Fridell JA, Agarwal A, Milgrom ML, Goggins WC, Murdock P, Pescovitz MD. Comparison of histidine-tryptophan-ketoglutarate solution and University of Wisconsin solution for organ preservation in clinical pancreas transplantation. Transplantation. 2004; 77(8):1304-6.

[31] Hesse UJ, Troisi R, Jacobs B, Berrevoet F, De Laere S, de Hemptinne B. Pancreas preservation with HTK solution in the pig. Transplant Proc. 1997; 29(8):3522-3.

[32] Hesse UJ, Troisi R, Jacobs B, Berrevoet F, De Laere S, Maene L, Vanden Broucke C, de Hemptinne B. Cold preservation of the porcine pancreas with histidine-tryptophan-ketoglutarate solution. Transplantation. 1998; 66(9):1137-41.

[33] Rubinsky B. Principles of low temperature cell preservation. Heart Fail Rev. 2003; 8(3):277-84.

[34] Kneteman NM, DeGroot TJ, Warnock GL, Rajotte RV. The evaluation of solutions for pancreas preservation prior to islet isolation. Horm Metab Res Suppl. 1990;25:4-9.

[35] Urushihara T, Sumimoto R, Sumimoto K, Jamieson NV, Ikeda M, Ito H, Hong HQ, Fukuda Y, Dohi K. Prolonged rat pancreas preservation using a solution with the combination of histidine and lactobionate. Transpl Int. 1992;5 Suppl 1:S336-9.

[36] Ploeg RJ, Boudjema K, Marsh D, Bruijn JA, Gooszen HG, Southard JH, Belzer FO. The importance of a colloid in canine pancreas preservation. Transplantation. 1992; 53(4):735-41.

[37] Morel P, Moss A, Schlumpf R, Nakhleh R, Lloveras JK, Field MJ, Condie R, Matas AJ, Sutherland DE. 72-hour preservation of the canine pancreas: successful replacement of hydroxyethylstarch by dextran-40 in UW solution. Transplant Proc. 1992; 24(3):791-4.

[38] Stratta RJ, Taylor RJ, Gill IS. Pancreas transplantation: a managed cure approach to diabetes. Curr Probl Surg. 1996; 33(9):709-808.

[39] Kenmochi T, Miyamoto M, Sasaki H, Une S, Nakagawa Y, Moldovan S, Benhamou PY, Brunicardi FC, Tanaka H, Mullen Y. LAP-1 cold preservation solution for isolation of high-quality human pancreatic islets. Pancreas. 1998; 17(4):367-77.

[40] Kehrer JP. Free radicals as mediators of tissue injury and disease. Crit Rev Toxicol. 1993;23(1):21-48.

[41] Kenmochi T, Miyamoto M, Une S, Nakagawa Y, Moldovan S, Navarro RA, Benhamou PY, Brunicardi FC, Mullen Y. Improved quality and yield of islets isolated from human pancreata using a two-step digestion method. Pancreas. 2000; 20(2):184-90.

[42] Vega JD, Ochsner JL, Jeevanandam V, McGiffin DC, McCurry KR, Mentzer RM Jr, Stringham JC, Pierson RN 3rd, Frazier OH, Menkis AH, Staples ED, Modry DL, Emery RW, Piccione W Jr, Carrier M, Hendry PJ, Aziz S, Furukawa S, Pham SM. A multicenter, randomized, controlled trial of Celsior for flush and hypothermic storage of cardiac allografts. Ann Thorac Surg. 2001; 71(5):1442-7.

[43] Cavallari A, Cillo U, Nardo B, Filipponi F, Gringeri E, Montalti R, Vistoli F, D'amico F, Faenza A, Mosca F, Vitale A, D'amico D. A multicenter pilot prospective study comparing Celsior and University of Wisconsin preserving solutions for use in liver transplantation. Liver Transpl. 2003; 9(8):814-21.

[44] Baldan N, Toffano M, Cadrobbi R, Codello L, Calabrese F, Bacelle L, Rigotti P. Kidney preservation in pigs using celsior, a new organ preservation solution. Transplant Proc. 1997; 29(8):3539-40.

[45] Baldan N, Rigotti P, Furian L, Valente ML, Calabrese F, Di Filippo L, Parise P, Sarzo G, Frison L, Ancona E. Pancreas preservation with Celsior solution in a pig autotransplantation model: comparative study with University of Wisconsin solution. Transplant Proc. 2001; 33(1-2):873-5.

[46] Uhlmann D, Armann B, Ludwig S, Escher E, Pietsch UC, Tannapfel A, Teupser D, Hauss J, Witzigmann H. Comparison of Celsior and UW solution in experimental pancreas preservation. J Surg Res. 2002; 105(2):173-80.

[47] Boggi U, Vistoli F, Del Chiaro M, Signori S, Croce C, Pietrabissa A, Berchiolli R, Marchetti P, Prato SD, Mosca F. Pancreas preservation with University of Wisconsin and Celsior solutions: a single-center, prospective, randomized pilot study. Transplantation. 2004; 77(8):1186-90.

[48] Kuroda Y, Kawamura T, Suzuki Y, Fujiwara H, Yamamoto K, Saitoh Y. A new, simple method for cold storage of the pancreas using perfluorochemical. Transplantation. 1988; 46(3):457-60.

[49] Riess JG. Fluorocarbon-based in vivo oxygen transport and delivery systems. Vox Sang. 1991;61(4):225-39.

[50] Kuroda Y, Fujino Y, Morita A, Tanioka Y, Ku Y, Saitoh Y. Oxygenation of the human pancreas during preservation by a two-layer (University of Wisconsin solution/perfluorochemical) cold-storage method. Transplantation. 1992; 54(3):561-2.

[51] Kuroda Y, Tanioka Y, Morita A, Hiraoka K, Matsumoto S, Fujino Y, Ku Y, Saitoh Y, Sugihara J, Okumura S, et al. The possibility of restoration of human pancreas function during preservation by the two-layer (University of Wisconsin solution/perfluorochemical) method following normothermic ischemia. Transplantation. 1994; 57(2):282-5.

[52] Kuroda Y, Hiraoka K, Tanioka Y, Matsumoto S, Morita A, Fujino Y, Suzuki Y, Ku Y, Saitoh Y. Role of adenosine in preservation by the two-layer method of ischemically damaged canine pancreas. Transplantation. 1994; 57(7):1017-20.

[53] Hiraoka K, Trexler A, Eckman E, Stage A, Nevile S, Sageshima J, Shibata S, Sutherland DE, Hering BJ. Successful pancreas preservation before islet isolation by the simplified two-layer cold storage method. Transplant Proc. 2001; 33(1-2):952-3.

[54] Ricordi C, Fraker C, Szust J, Al-Abdullah I, Poggioli R, Kirlew T, Khan A, Alejandro R. Improved human islet isolation outcome from marginal donors following addition of oxygenated perfluorocarbon to the cold-storage solution. Transplantation. 2003; 75(9):1524-7.

[55] Matsumoto S, Qualley SA, Goel S, Hagman DK, Sweet IR, Poitout V, Strong DM, Robertson RP, Reems JA. Effect of the two-layer (University of Wisconsin solution-perfluorochemical plus O2) method of pancreas preservation on human islet isolation, as assessed by the Edmonton Isolation Protocol. Transplantation. 2002; 74(10):1414-9.

[56] Brandhorst D, Iken M, Brendel MD, Bretzel RG, Brandhorst H. Successful pancreas preservation by a perfluorocarbon-based one-layer method for subsequent pig islet isolation. Transplantation 2005; 79: 433.

[57] Mirbolooki M, Lakey JR. Oxygen and pancreas preservation. Transplantation. 2006; 81(3):492

[58] Papas KK, Hering BJ, Gunther L, Rappel MJ, Colton CK, Avgoustiniatos ES. Pancreas oxygenation is limited during preservation with the two-layer method. Transplant Proc. 2005; 37(8):3501-4.

[59] Kin T, Mirbolooki M, Salehi P, Tsukada M, O'Gorman D, Imes S, Ryan EA, Shapiro AM, Lakey JRT. Islet isolation and transplantation outcomes of pancreas preserved with University of Wisconsin solution versus two-layer method using pre-oxygenated perfluorocarbon. Transplantation . 2006: 82(10):

[60] Belzer FO, Ashby BS, Gulyassy PF, Powell M. Successful seventeen-hour preservation and transplantation of human-cadaver kidney. N Engl J Med. 1968;278(11):608-10.

[61] Florack G, Sutherland DE, Heil J, Squifflet JP, Najarian JS. Preservation of canine segmental pancreatic autografts: cold storage versus pulsatile machine perfusion. J Surg Res. 1983 May;34(5):493-504.

[62] Taylor MJ, Baicu S, Leman B, Greene E, Vazquez A, Brassil J. Twenty-four hour hypothermic machine perfusion preservation of porcine pancreas facilitates processing for islet isolation. Transplant Proc. 2008 Mar;40(2):480-2.

[63] Leeser DB, Bingaman AW, Poliakova L, Shi Q, Gage F, Bartlett ST, Farney AC. Pulsatile pump perfusion of pancreata before human islet cell isolation. Transplant Proc. 2004 May;36(4):1050-1.

[64] Minor T, Saad S, Kitting M, et al. Short term restoration by gaseous oxygen for long term preservation of predamaged livers from non heart beating donors. Transplant Proc 1997; 29: 3474

[65] Rolles K, Foreman J, Pegg DE. Preservation of ischemically injured canine kidneys by retrograde oxygen persufflation. Transplantation 1984; 38: 102

[66] Stubenitsky BM, Ametani M, Danielewicz R, Southard JH, Belzer FO. Regeneration of ATP in kidney slices after warm ischemia and hypothermic preservation. Transpl Int. 1995;8(4):293-7.

[67] Fuller BJ, Busza AL, Proctor E. Possible resuscitation of liver function by hypothermic reperfusion in vitro after prolonged (24-hour) cold preservation--a 31P NMR study. Transplantation. 1990 Sep;50(3):511-3.

[68] Tago M, Seno S, Bando K, Teraoka H, Senoo Y, Teramoto S. [High energy phosphates and mitochondrial respiratory function after 24 hours cardiac preservation--comparison between simple hypothermic immersion and continuous hypothermic perfusion] Kokyu To Junkan. 1989 Jan;37(1):61-3.

[69] Fuller BJ, Lee CY. Hypothermic perfusion preservation: the future of organ preservation revisited? Cryobiology. 2007 Apr;54(2):129-45.

[70] Bogardus GM, Schlosser RJ. The influence of body temperature upon ischemic renal damage. Surgery 39 (6):970-4

[71] Belzer FO, Southard JH. Principles of solid-organ preservation by cold storage. Transplantation. 1988; 45(4):673-6.

[72] Fahy GM, MacFarlane DR, Angell CA, Meryman HT. Vitrification as an approach to cryopreservation. Cryobiology. 1984 Aug;21(4):407-26.

[73] Belzer F.O. In Organ preservation: Basic and applied principles, Pegg DE, Jacobson IA, Halasz NA. Eds.; MTP press: Lancaster, 1982; pp 333.

[74] Winchell RJ, Halasz NA. The effects of cooling rates and storage temperature on the function of 24-hour cold-preserved rabbit kidneys. Transplantation. 1988; 46(6):918-9.

[75] Fuller BJ, Attenburrow VD, Newsome C. Experimental studies on continuous hypothermic liver perfusion with a synthetic solution containing gelatin polypeptides (haemaccel). Cryobiology. 1978 Jun;15(3):279-89.

[76] Lakey JR, Wang LC, Rajotte RV. Optimal temperature in short-term hypothermic preservation of rat pancreas. Transplantation. 1991; 51(5):977-81.

[77] Inui H, Kwon AH, Yoshida K, Tsuchiya H, Inoue K, Kamiyama Y. Cold preservation of rat pancreatic islets just above the freezing point using University of Wisconsin solution. Pancreas 2001;23(4):382-6.

[78] Martin DR, Scott DF, Downes GL, Belzer FO. Primary cause of unsuccessful liver and heart preservation: cold sensitivity of the ATPase system. Ann Surg. 1972; 175(1):111-7.

[79] Wang LCH. Ecological, physiological and biochemical aspects of torpor in mammals and birds. In: Advances in comparative and environmental physiology, Wang L, Ed.; Springer: Berlin, 1989; pp. 361

[80] Plesnila N, Muller E, Guretzki S, Ringel F, Staub F, Baethmann A. Effect of hypothermia on the volume of rat glial cells. J Physiol. 2000; 523 Pt 1:155-62.

[81] Hochachka PW. Defense strategies against hypoxia and hypothermia. Science. 1986; 231(4735):234-41.

[82] Boutilier RG. Mechanisms of cell survival in hypoxia and hypothermia. J Exp Biol. 2001; 204(Pt 18):3171-81.

[83] Kellum JA, Song M, Li J. Lactic and hydrochloric acids induce different patterns of inflammatory response in LPS-stimulated RAW 264.7 cells. Am J Physiol Regul Integr Comp Physiol. 2004; 286(4):R686-92.

[84] Mirbolooki M, Lakey J. Pancreas preservation for islet isolation. In: Islet transplantation and beta cell replacement therapy. Shapiro AMJ, Shaw JAM (eds.) informa healthcare USA Inc. New York 2007 pp 101

[85] Palombo JD, Pomposelli JJ, Hirschberg Y, Blackburn GL and Bistrian BR, Glycolytic support of adenine nucleotides in rat liver flush-preserved with UW or Collins' II. Importance of donor nutritional status. Transplantation 1989; 48: 901 905.

[86] Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex.Mol Cell. 1999 Mar;3(3):287-96.

[87] Antonsson B, Martinou JC. The Bcl-2 protein family. Exp Cell Res. 2000 Apr 10;256(1):50-7.

[88] Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schröter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschopp J. Inhibition of death receptor signals by cellular FLIP. Nature. 1997 Jul 10;388(6638):190-5.

[89] Emamaullee JA, Shapiro AM. Interventional strategies to prevent beta-cell apoptosis in islet transplantation. Diabetes. 2006;55(7):1907-14.

[90] Wang J, Lenardo MJ. Roles of caspases in apoptosis, development, and cytokine maturation revealed by homozygous gene deficiencies. J Cell Sci. 2000 Mar;113 (Pt 5):753-7.

[91] Emamaullee JA, Rajotte RV, Liston P, Korneluk RG, Lakey JR, Shapiro AM, Elliott JF. XIAP overexpression in human islets prevents early posttransplant apoptosis and reduces the islet mass needed to treat diabetes. Diabetes. 2005 Sep;54(9):2541-8.

[92] Anderson CD, Belous A, Pierce J, Nicoud IB, Knox C, Wakata A, Pinson CW, Chari RS. Mitochondrial calcium uptake regulates cold preservation-induced Bax translocation and early reperfusion apoptosis. Am J Transplant. 2004 Mar;4(3):352-62.

[93] Cau J, Favreau F, Tillement JP, Lerman LO, Hauet T, Goujon JM. Trimetazidine reduces early and long-term effects of experimental renal warm ischemia: a dose effect study. J Vasc Surg. 2008 Apr;47(4):852-860.

[94] http://www.biozentrum.uni-frankfurt.de/Pharmakologie/EU-Web/Bilder/Oxstress.jpg

[95] Fuller BJ. The effects of cooling on mammalian cells. In: Fuller B, Grout B, eds. Clinical applications of cryobiology. London: CRC, 1991.

[96] Lakey JR, Tsujimura T, Shapiro AM, Kuroda Y. Preservation of the human pancreas before islet isolation using a two-layer (UW solution-perfluorochemical) cold storage method. Transplantation 2002; 74: 1809-11.

[97] Clark LC Jr, Gollan F. Survival of mammals breathing organic liquids equilibrated with oxygen at atmospheric pressure. Science. 1966 ;152(730):1755-6

[98] Schmidt-Nielsen K. Respiration in water. In: Animal Physiology. Adaptation and Environment, 3rd edn. New York, NY: Cambridge University Press, 1983: 1-25.

[99] O'Brien RN, Langlais AJ, Seufert WD. Diffusion coefficients of respiratory gases in a perfluorocarbon liquid. Science. 1982 ;217(4555):153-5.

[100] McCabe M, Laurent TC. Diffusion of OXYGEN, Nitrogen and water in hyluronate solutions. Biochim Biophys Acta. 1975 Jul 14;399(1):131-8.

[101] Brandhorst D, Bretzel RG, Brandhorst H. Oxygen and pancreas preservation Transplantation. 2006 ;81(3):492

[102] McCabe MG, Maguire DJ, Bourgain R. The flux of oxygen within tissues. Adv Exp Med Biol. 2003;540:291-5.

---

[103] MacDougall JD, McCabe M. Diffusion coefficient of oxygen through tissues. Nature. 1967 Sep 9;215(5106):1173-4.

[104] McCabe M. The solubility of oxygen in erythrocyte ghosts and the flux of oxygen across the red cell membrane. Adv Exp Med Biol. 1986;200:13-20.

[105] Gold HJ. Kinetics of facilitated diffusion of oxygen in tissue slices : J Theor Biol. 1969 Jun;23(3):455-62

[106] Longmuir IS, Sun S. A hypothetical tissue oxygen carrier. : Microvasc Res. 1970 Jul;2(3):287-93.

[107] Zeigler DM. Role of reversible oxidation-reduction of enzyme thiols-disulphides in metabolic regulation Ann Revs Biochem , 1985; 54:305-308

[108] Bentley TB, Meng H, Pittman RN. Temperature dependence of oxygen diffusion and consumption in mammalian striated muscle. Am J Physiol. 1993 ;264(6 Pt 2):H1825-30

[109] Ellsworth ML, Pittman RN. Heterogeneity of oxygen diffusion through hamster striated muscles Am J Physiol. 1984;246(2 Pt 2):H161-7

[110] Desaulniers N, Moerland TS, Sidell BD. High lipid content enhances the rate of oxygen diffusion through fish skeletal muscle Am J Physiol. 1996 ;271(1 Pt 2):R42-7

[111] Matsumoto S, Kuroda Y, Hamano M, Kim Y, Suzuki Y, Ku Y, Saitoh Y. Direct evidence of pancreatic tissue oxygenation during preservation by the two-layer method Transplantation. 1996 ;62(11):1667-70

[112] Papas KK, Hering BJ, Guenther L, Rappel MJ, Colton CK, Avgoustiniatos ES. Pancreas oxygenation is limited during preservation with the two-layer method Transplant Proc. 2005 ;37(8):3501-4.

[113] Avgoustiniatos ES, Hering BJ, Papas KK. The rat pancreas is not an appropriate model for testing the preservation of the human pancreas with the two-layer method Transplantation. 2006 ;81(10):1471-2

[114] Lakey JR, Tsujimura T, Shapiro AM, Kuroda Y. Preservation of the human pancreas before islet isolation using a two-layer (UW solution-perfluorochemical) cold storage method. Transplantation. 2002; 74(12):1809-11.

Chapter Two

Methodology

Rationale & Design

Animals

Male Sprague-Dawley rats (weight: 200-250 g, pancreas donors) were obtained from The Health Sciences Laboratory Animal Services (HSLAS) at the University of Alberta. Sprague Dawley rats are an outbred strain of albino, laboratory rats belonging to the species *Rattus norvegicus*. They are originally developed from the Wistar rat strain and used widely for experimental purposes because of their calmness and ease of handling. The adult body weight is 250-300g (female), and 450-520g (male). The typical life span is 2.5 - 3.5 years. All experiments were conducted in accordance with the regulations and policies of the Canadian Council on Animal Care and approved by the Research Ethics Board of the University of Alberta.

Surgical Procedures

Pancreata were procured according to standardized procurement procedure. In experiments presented in chapters 3 and 4, rats received inhalational 4% halothane followed by 1.5% halothane to maintain anesthesia. In the experiments presented in chapters 5 and 6 Isoflurane replaced halothane as anaesthesia could be induced more rapidly than with halothane. A midline laparotomy was performed and the pancreas was exposed. The lower end of the bile duct was clamped at the bile duct and duodenum intersection and upper end of it at a point prior to where it branches off to the liver (Figure 2-1). The bile duct was cannulated with a PE 50 tubing attached to a 23 gauge needle attached to a 10cc syringe (Figure 2-1). Pancreas was expanded gently with injecting 10-20 mL of cold UW/HBSS solution into the duct and detached from all connections with a downward brushing motion, using the blunt ends of the scissors. Spleen was used as a marker and kept attached during preservation. The pancreas was washed in either HBSS (chapters 3 & 4) or UW solution (chapters 5 & 6) and then transferred to 50 ml tubes for 24 hours preservation.

Figure 2-1: Rat pancreas procurement

Experimental Groups

The pancreata were stored in 20 mL of each respective solution and stored on ice for 24 hours. Freshly isolated (FI) tissue was obtained from a separate group of animals to provide an approximation of *in vivo* levels for all parameters assessed. The experimental groups are as follows:

Experiment 1:

Pancreata were removed after 20 mL intraductal injection of:

- Cold Hank's Balanced Salt Solution (HBSS)
- Preoxygenated solutions of HBSS
- Preoxygenated PFC
- Preoxygenated ePFC

and then preserved in HBSS for 24 hours.

Experiment 2:

Pancreata were removed after 20 mL intraductal injection of HBSS, and preserved for 24 hours in:

- HBSS
- HBSS with continuous bubbling of oxygen

Experiment 3:

Pancreata were removed after 20 mL intraductal injection of filtered UW, and preserved for 24 hours in:

- UW
- TLM
- UW + continuous bubbling of oxygen
- TLM + continuous bubbling of oxygen

Experiment 4:

Pancreata were removed after 10 mL intraductal injection of filtered UW, and preserved for 24 hours in:

- UW
- UW + 5mM Glucose
- UW + continuous bubbling of oxygen
- UW + continuous bubbling of oxygen + 5mM Glucose

Partial Oxygen Pressure

The partial oxygen pressure in PFC was measured at the end of TLM preservation using a Foxy fiber optic oxygen sensor (Ocean Optics, Inc., FL). The sensor uses the fluorescence of a ruthenium complex in a sol-gel to measure the partial pressure of oxygen. To calibrate the sensor, we used 100% O_2, air as 21% O_2, and Nitrogen as 0%

O_2. Calibration was done by passing the gas through a coil placed in ice. The software could adjust the oxygen content by the temperature variations within 3°C.

Tissue Sampling and Time Points

Tissue samples (100 mg) were collected initially and at 1, 4, 12, and 24 hours after procurement. All the samples were collected from the surface of the tissue to meet the assumption of having 100%-oxygenated pancreatic tissue. To arrest metabolic activity, samples were snap-frozen in liquid nitrogen and subsequently stored at -80 °C until processed. Frozen samples (100 mg) were grinded in perchloric acid (6%) containing 1 mM of EDTA. A sample (50 µl) from homogenate was placed into 950 µl of a 0.15M NaOH solution for later protein assay. The precipitated protein was removed by centrifugation (20 min at 20,000 g). Acid extracts were neutralized by the addition of 3 M KOH/0.4 M Tris/0.3 M KCl and then re-centrifuged. Aliquots of neutralized extracts were immediately processed via standard enzyme-linked metabolite assays.

Adenosine Tri-, Di-, and Mono-Phosphate Assays

In a 96 well plate, 100 uL of aliquots of neutralized extracts were plated. To make the ATP reaction mixture, TRIS buffer (pH = 8.0) containing NAD+ (1.0 mM); Magnesium Sulfate solution (1.5 ml of 100mM solution); Glucose (2.0mM); ADP (0.5 mM); and G6PDH (0.5 units per assay) was used. Spectrophotometric analysis was performed to measure the initial absorbance of NADH at 340 nm using a Dynex MRX TC plate reader. Hexokinase (0.5 units per assay) was added to the wells and the plate was reassessed every hour (approx. 3-5 hours) to completion of the reactions below.

$$\text{Glucose-6-P} + \text{NAD}^{+} \xrightarrow[\text{Dehydrogenase}]{\text{Glucose-6-P}} \text{6-P-gluconolactone} + \text{NADH}$$

$$\text{ATP + glucose} \xrightarrow{\text{Hexokinase}} \text{ADP + glucose-6-Phosphate}$$

In another 96 well plate, 100 uL of aliquots of neutralized extracts were plated. To make the ADP/AMP reaction mixture, Imidazole buffer (pH = 7.0) containing NADH (0.4 mM), PEP (0.5mM), ATP (0.4mM), 1.5 ml MgSO4 (100mM solution), 3.0 ml KCl (500mM solutionn), and LDH (0.5 units/assay) was used. Spectrophotometric analysis was performed to measure the initial absorbance of NADH. Pyruvate Kinase (0.5 units/assay) was added. The plate was reassessed every 15 minutes (approx. 1 hour) to completion of the ADP reaction. Myokinase (0.5 units/assay) was added and plate was reassessed every hour to completion of the AMP reaction.

$$\text{Pyruvate + NADH + H}^{+} \xrightarrow[\text{Dehydrogenase}]{\text{Lactate}} \text{lactate + NAD}^{+}$$

$$\text{ADP + P-pyruvate} \xrightarrow[\text{Kinase}]{\text{Pyruvate}} \text{ATP + pyruvate}$$

$$\text{AMP + ATP} \xrightarrow{\text{Myokinase}} \text{2ADP}$$

The changes in optical density due to ATP, ADP, and AMP utilization were calculated using the Microplate Analysis Program (version 1.75). The energy charge (EC) was calculated according to Atkinson [1]: EC = (ATP + 0.5 ADP)/(ATP + ADP + AMP).

Lactate

L-lactate is constantly produced from pyruvate via the enzyme lactate dehydrogenase (LDH) in a process of fermentation during normal metabolism and exercise. Under anaerobic conditions, glycolysis remains the main pathway in ATP production which leads to accumulation of lactate in the tissue. To prepare Hydrazine buffer reaction mixture (pH = 9.6), Hydrazine (150 mM) and NAD+ (1.0Mm) were dissolved in distilled water. NaOH nuggets (approx. 5) and a few drops of 2M NaOH solution were used to bring the pH to 9.6. Sample extracts were pipetted into wells. 200 ul of Hydrazine buffer was added to each well. Plate was read for lactate initial (340nM—Metabolite assay). One unit per assay LDH (from bovine heart) was added. Plate was read every 1 hour for approximately 4 hours until the reaction was completed.

$$\text{Pyruvate} + \text{hydrazine} \rightarrow \text{pyruvate hydrazone}$$

$$\text{Lactate} + \text{NAD}^+ \xrightarrow[\text{dehydrogenase}]{\text{lactate}} \text{pyruvate} + \text{NADH} + \text{H}^+$$

The changes in optical density were calculated using the Microplate Analysis Program.

Malondialdehyde (MDA)

This assay was modified slightly from the original according to Yagi [2,3], however, supporting references justify all modifications. To make the reaction mixture, 0.2 ml of 7 % sodium dodecyl sulfate (SDS), 2.0 ml of 0.1 M HCl, 0.3 ml of 10 % Phosphotungstic acid, and 1.0 ml of 0.67 % TBA (2-Thiobarbituric acid) were mixed.

The assay was done according to the following protocol:

1) Add 1 ml of PBS (K_2HPO_4, 50 mM) to 100 mg of frozen tissue
2) Homogenize (3 × 5 sec) the tissues and put them in ice
3) Spin for 15 min at 15000 g at 0-5°C
4) Take 500 μl of the supernatant and transfer into eppendorf tubes
5) Add 875 μl of the reaction mixture to each tube and vortex
6) Boil for 1 hr at 95°C.
7) Add 750 μl N-Butanol and vortex.

8) Spin in 1000 g for 1 min.

9) Take 100 μl and read it in duplicate (Excitation: 530/25, Emission: 575/15).

The standards used in this assay were prepared as follows:

1) Mix 100 μl of concentrated 1,1,3,3 tetraethoxypropane (malonaldehyde Bis diethyl acetal) with 4.9 ml of 0.1 M HCl (you have to prepare each day).

2) Incubate at 37°C (water bath) for 15 min

3) Dilute this 1:1000 (10 μl made up to 10 ml PBS) to give 122 μM MDA.

4) Make the standards (16, 8, 4, 2, 1, 0 μM), treat them exactly like the samples.

Glutathione

Re-oxygenation of tissues leads to the generation of oxygen free radicals (OFR) which further potentiates cellular injury [4]. A primary endogenous cellular defense mechanism is the reduction of OFRs via glutathione peroxidase and glutathione (reduced) [5]. The tripeptide glutathione (GSH) is an integral component of this defense mechanism against cellular oxidative stress injury. We quantitatively measured Reduced Glutathaione (GSH) using monochlorobimane [6].

Solutions and Enzyme

- Imidazole Buffer (100mM) and pH=7.0 as the solvent and buffer.
- Monochlorobimane: 25 mg of MCBM in the vial was dissolved in 2.2 ml of absolute ethanol (final concentration of 50 mM) and stored in -70 freezer in 150 μL aliquots.
- GSH for Standard: 1 mM standard was made using Imidazole and stored in -70 freezer.

Reaction mixture (150 μL for each well) was made adding 100 μL of MCBN in 15 ml Imidazole buffer. It is light sensitive. Sample extract was prepared as discussed in sample preparation procedure form. Standards were prepared in 0, 10, 25, 50, 100, 200 μM concentration.

The assay was done according to the following protocol:

1. Take 100 μL of samples and standards (3 columns)
2. Add 150 μL of reaction mixture to each well.
3. Read by fluorometer (wave length: Excitation 380/20 nm – Emission: 485/20 nm).
4. Add 0.1 Unit/assay of GST
5. Incubate the plate in a dark place and read every 30 min untill completion of the reaction (about 2 hours).
6. Call the last read as GSH final.

The changes in optical density were calculated using the Microplate Analysis Program.

DNA Fragmentation

The assay is based on the quantitative "sandwich enzyme immunoassay" principle using mouse monoclonal antibodies directed against DNA and histones. The samples are placed into a streptavidin-coated microplate and incubated with a mixture of anti-histone-biotin and anti-DNA-peroxidase (POD) antibodies. During the incubation interval, nucleosomes is captured via their histone component by the anti-histone-biotin antibody, while binding to the streptavidin-coated microplate. Simultaneously, anti-DNA-peroxidase binds to the DNA part of the nucleosomes. After removal of the unbound antibodies, the amount of peroxidase retained in the immunocomplex is photometrically determined with ABTS as the substrate. As compared to Comet Assay and laddering by gel electerophoresis, this is a quantitative and more sensitive assay.

Preparation of Working Solution

1. Anti-Histone Biotin and Anti-DNA-POD antibodies: the lyophilizated powders were reconstituted in 450 μl double distilled water for 10 min.

2. ABTS (2,2 -Azino-di[3-ethyl-benz-thiazolin-sulfonat]) solution: Depend on the number of sample tested, each tablet was dissolved in 5 ml of substrate solution. ABTS solution can be used only for 1 month And should be protected from light.

For each well, 4µl of prepared Anti-Histone Biotin, 4µl of prepared Anti-DNA POD and 72µl of fresh Incubation buffer as the immunoreagent mixture were mixed.

The assay was done according to the following protocol:

1. Add 20 µl of sample and controls to microplate.
2. Add 80 µl of the immunoreagent solution to each well.
3. Cover the microplate with cover foil and incubate it on a shaker under gently shaking (300 rpm) for 2 h at room temperature.
4. Remove the solution thoroughly by tapping or suction.
5. Rinse each well 3 times with 250-300 µl incubation buffer and remove the solution carefully.
6. Pipette 100 µl ABTS solution to each well.
7. Incubate on a plate shaker at 250 rpm until color development is sufficient for a colorimetric analysis (approx. after 10-20 min).
8. Read at 405 nm.

Caspase 3

Caspases (Cysteine aspartic acid protease) are a family of proteases that mediate cell death and are important to the process of apoptosis. Caspase 3 (also referred to as CPP32, Yama, and apopain) is a member of the *Caenorhabditis elegans Death protein-3* (CED-3) subfamily of caspases and is one of the critical enzymes of apoptosis. Caspase 3 plays a central role in mediating nuclear apoptosis including chromatin condensation and DNA fragmentation as well as cell blebbing. The caspase 3

(Sigma) colorimetric assays are based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by these caspases, resulting in the release of the p-nitroaniline (pNA) moiety.

$$\text{Ac-DEVD-pNA} \xrightarrow{\text{Caspase 3}} \text{Ac-DEVD} + \text{pNA}$$

p-Nitroaniline has a high absorbance at 405 nm. The concentration of the pNA released from the substrate was calculated from the absorbance values at 405 nm.

The assay was done according to the following protocol:

- Dilute Assay Buffer 10-fold with 17 megohm water to the final concentrations of 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4, 2 mM EDTA (ethylenediaminetetraacetic acid), 0.1% CHAPS (3[(3-Cholamidopropyl) dimethylammonio]-propanesulfonic acid), and 5 mM DTT (Dithiothreitol).
- Dissolve the vial contents (15 mg) in 1.2 ml of DMSO to prepare a 20 mM Caspase 3 substrate (Ac-DEVD-pNA) stock solution. For assays using 96 well plates dilute the 20 mM stock solution to 2 mM with Assay Buffer.
- Dissolve the vial in 0.72 ml DMSO to make p-Nitroaniline standard for developing a calibration curve for assays in 96 well plate. Store the stock solution at –20 °C.
- Homogenize (3 × 5 sec) the tissues with 1 ml of PBS and put them in ice
- Centrifuge the homogenate and transfer the supernatant to eppendorf tube.
- Centrifuge the eppendorf tube at 16,000 to 20,000 x *g* for 10 to 15 minutes at 4 °C.
- Transfer the supernatants to new tubes.
- Analyze the supernatant immediately or freeze in liquid nitrogen and store in aliquots at –70 °C.
- Place 5 µl of each sample in the appropriate wells
- Add 85 ul of Assay Buffer to each of the wells
- Start the reaction by adding 10 µl of caspase 3 substrate to each well

- Read initial Absorbance at 405 nm.
- Mix it gently by shaking. Try to avoid forming bubbles in the wells.
- Cover the plate and incubate at 37 °C for 70 to 90 minutes. If signal is too low, continue the incubation overnight.
- Read final Absorbance at 405 nm.
- Calculate the changes in optical density.

Caspases 8 & 9

Caspases 8 and 9 play central roles in extrinsic and intrinsic pathways of apoptosis, respectively. The assays (Biovision kits) are based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labeled substrate Ile-Glu-Thr-Asp-pNA (IETD-*p*NA) by caspase 8 and labeled substrate Leu-Glu-His-Asp-pNA (LEHD-*p*NA) by caspase 9.

$$\text{IETD-pNA} \xrightarrow{\text{Caspase 8}} \text{IETD + pNA}$$

$$\text{LEHD-pNA} \xrightarrow{\text{Caspase 9}} \text{LEHD + pNA}$$

The assay was done according to the following protocol:

- Protect IETD-*p*NA from light.
- Homogenize (3 × 5 sec) the tissues with 1 ml of PBS and put them in ice
- Centrifuge the homogenate and transfer the supernatant to eppendorf tube.
- Centrifuge the eppendorf tube at 16,000 to 20,000 x *g* for 10 to 15 minutes at 4 °C.
- Transfer the supernatants to new tubes.
- Add 50 µl of samples to appropriate well for each assay.
- Add 50 µl of Reaction Buffer (containing 10 mM DTT) to each sample.

- Add 5 µl of the 4 mM IETD-*p*NA substrate for caspase 8 plate and 4 mM LEHD-*p*NA in caspase 9 plate (200 µM final conc.).
- Read initial value at 400- or 405-nm in a microtiter plate reader
- Incubate at 37°C for 1-2 hour.
- Read final value at 400- or 405-nm in a microtiter plate reader
- Calculate the changes in optical density.

Protein Assay

To standardize metabolite data, values were reported in terms of 'per gram protein'. Protein was measured according to Lowry et al [7].

Sample preparation was done according to the following protocol:

A) Grind tissue sample (100mg) in 6% Perchloric acid (PCA) and immediately remove 50 µl of sample from homogenate.

B) Place into 950 µl of a 0.15M NaOH solution (note: protein will be safe in basic (solution)

C) Mix well – be certain diluted sample containing homogenate is clear enough for test.

D) Pipette 30 µl of sample into wells and test according to protein assay protocol (below)

Solutions

LOWRY "A": Take 0.5000 g of cupric sulfate, pentahydrate (MW=249.7 g/mol) and 1.000 g of Sodium citrate (MW=294.1 g/mol) to a 100 mL graduated cylinder, and dissolve using distilled water (d.w.). Dilute up to full volume (100 mL) using d.w.

LOWRY "B": To make 500 mL of Lowry "B", take 10.0 g of sodium carbonate and 2.000 g of sodium hydroxide and dissolve in d.w. Top up to full volume (500 mL) using a graduated cylinder and d.w.

4% BSA STOCK SOLUTION: Take 200 mg of BSA and weigh directly into a 5 mL volumetric flask. Add a very small amount of sterile saline and carefully swirl to dissolve. When BSA is dissolved, add enough sterile saline to bring solution up to full volume. Invert slowly several times to ensure proper mixing of solution— watch for bubbles!! Dilute 4% BSA to make a 0.04% solution of BSA by taking 10 µl of the 4% solution, and dilute it with 900 µL of sterile saline.

The assay was done according to the following protocol:

1. Make a 4% albumin stock solution for the protein assay. (Weigh out 200 mg of Bovine Serum Albumin (BSA) into a 5 ml volumetric flask, and fill to final volume with sterile saline.
2. Make a 0.04% standard solution of BSA by adding 20 µL of 4% solution, to 1980 µL of sterile saline. (2 mL total volume is sufficient for standards 10, 20, 30, 40, 50, 100 µL in triplicate)
3. Pipette 20 µL of the diluted homogenate mixture into a microwell plate.
4. Pipette the BSA standard (in triplicate), into the remaining wells
5. Add 200 µL of Reaction mixture to each well. Be certain to mix each well using a 200 µL multi-pipettor. (REACTION MIX: Take 1.0 mL of solution A: (0.5% $CuSO_4$ + 1% sodium citrate) and add 49 mL of solution B: (2% Na_2CO_3 + 0.1M NaOH)
6. Leave plates for 10 minutes.
7. Add 20 µL of Folin reagent to each well, and mix well (very important) –use 200 µL multi-pipettor again.
8. Leave plates for an additional 30 minutes.

9. Read plates using plate reader, with 750 nm filter on metabolite assay (protein assay)

Histology

Biopsies of pancrteatic tissues were fixed in 10% buffered formalin and embedded in paraffin. Serial sections 4.5 µm thick were cut. Deparaffinized sections were stained for structure (Hematoxylin & Eosin), for β-cells (insulin-positive), and for α-cells (Glucagon-positive) by an immunoperoxidase technique according to the following protocol.

Embedding fixed tissue sample in paraffin was done according to the following protocol:

1. Remove cassettes containing fixed, tissue samples from processing unit.
2. Place cassettes into paraffin bath (temperature at 58 and 63 °C).
3. Place cassette onto warming tray, and open to remove tissue.
4. Dispense a small amount of paraffin into the bottom of the metal molding tray, and then quickly position tissue sample into the base of the tray, using heated forceps.
5. Place cassette base onto the top of the tray/sample/wax, and quickly dispense enough liquid paraffin to sufficiently cover the holes on top of the cassette.
6. Immediately place metal molding tray, with sample/cassette, onto a metal water bath surface—pre-cooled to between – 4 and –15 °C.
7. Once wax has completely solidified, pull cassette from metal molding tray.
8. Using a sharp knife, remove excess wax from edges of cassette.
9. Using a surgical razor blade, you may cut wax around sample in an angled fashion, to produce a faceted edge around the sample

10. Keep samples cold on refrigerated water bath surface, until just prior to placement into an alcohol/glycerol, block softening bath.

Microtome

11. Place cassette into the Microtome. Be certain there is a sharp blade installed in the microtome unit.
12. Position the block so that only a few rough, micro-thin slivers of the block are removed initially.
13. Begin cranking wheel of microtome.
14. Once a couple slices are cut, and you are satisfied with the depth you have cut into, gently grasp the end of the paraffin ribbon with two fingers, and gently support the ribbon while continuing to slice out about a 10 cm long ribbon of connecting tissue/paraffin slices.
15. Once you have obtained the ribbon, take either the rounded end of forceps, or a smooth wooden stick, to help support the ribbon as you move it to a water bath, preheated to 45°C (± 2 °C). Glass water bath holding approximately 1-1.5 L of water will also contain approximately 80-100 mL of diluted Chrom Alum gelatin solution.
16. Using forceps, remove first couple of slices of the ribbon by quickly touching blunt end to paraffin slices you wish to remove.
17. Gently float a pre-labeled slide angled with top end towards you, in order to attract slices of paraffin/tissue. Once paraffin/tissue end sticks to the slide, gently turn bottom end of slide upward. Paraffin/tissue sample should then stick to the slide.
18. Quickly view slide under the microscope (but do not let slide/sample dry out)! Ensure that sample is a good slice of what view you are interested in.
19. Repeat steps 13 through 18, until you are satisfied with your sample.

20. Place slide at an upright angle, in order to let excess water run off the slide.
21. Place slide containing sample, into a drying oven set to 50 °C. Leave slides in oven until completely dried (anywhere from 15 to 25 minutes). Slides are usually kept upright in a holding rack to assist drying process).

Dewaxing

22. Place slides containing samples into slide rack, and then place into xylene, dip for 1-2 minutes.
23. Dip slides into fresh xylene for an additional 1-2 minutes.
24. Dip slides into fresh xylene dip for a final 1-2 minutes.
25. Dip slides into absolute alcohol—for 15 dips
26. Dip slides into absolute alcohol—for 15 dips.
27. Dip slides into 95% alcohol—for 15 dips.
28. Dip slides into 95% alcohol—for 15 dips.
29. Dip slides into water rinse bucket—allow fresh water to run into bucket (while gently dipping slides) until all alcohol runs clear off slides.

Hematoxylin Staining

30. Place slides into Hematoxylin stain (Rat pancreas=1 min 10 sec.)
31. Rinse with water X2
32. Quickly dip each slide individually, into acid/alcohol (0.25%) then immediately rinse gently with water, to remove the dip.
33. Place slides into Scott's or Lithium carbonate dip—should see specimens on slide turn blue within a second or two. (2 dips)
34. Rinse with water X2

Eosin Staining

35. Place slides into Eosin stain (15 sec)–adjust as necessary according to amount of time placed into Hematoxylin stain. (15 dips approx.=15sec)

36. Place slides into 95% alcohol (15 dips).
37. Place slides into 95% alcohol (10 dips).
38. Place slides into fresh absolute alcohol (10 dips).
39. Place slides into fresh absolute alcohol (10 dips).
40. Place slides into a final, fresh absolute alcohol (10 dips).
41. Place slides into xylene to remove alcohol (15 dips).
42. Place slides into fresh xylene (10 dips).
43. Place slides into fresh xylene (10 dips).

Immunohistochemistry

Paraffin sections were rehydrated to water, followed by the quenching of endogenous peroxidases by a solution of 10% hydrogen peroxide in methanol for six minutes. The slides were then blocked in 20% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 15 minutes at room temperature, and then incubated with primary guinea pig anti-Insulin (1:1000, DAKO, Carpinteria, CA, USA) or mouse anti-Glucagon (1:5000, Sigma, St. Louis, Missouri, USA) antibodies for 30 minutes at room temperature.. Following washes in PBS, secondary biotinylated goat anti-guinea pig and biotinylated anti-mouse (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) antibodies was applied for 20 minutes at room temperature. After washes in PBS, sections were then incubated with the avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA) and (ABC)-enzyme complex with diaminobenzidine as chromagen (BioGenex, San Ramon, CA, USA). The sections were then counterstained with Harris Hematoxylin and Alcoholic Eosin Y, dehydrated, and coverslipped using Entellan mounting media (Electron Microscopy Sciences, Hattlefield, PA, USA).

Solution's pH

Solution's pH was measured by symphony SB21 pH meter (VWR Scientific products). The meter was calibrated with standard pH solutions purchased from Fischer Scientific.

Lactate Dehydrogenase (LDH) Release

Unlike many other cytoplasmic enzymes which exist in many cells either in low amount (e.g., alkaline and acid phosphatase) or unstable, LDH is a stable cytoplasmic enzyme present in all cells and rapidly released into the supernatant solution upon damage of the plasma membrane. LDH activity can be determined by a coupled enzymatic reaction: LDH oxidizes lactate to pyruvate which then reacts with tetrazolium salt to form formazan. The increase in the amount of formazan produced in supernatant directly correlates to the increase in the number of lysed cells. The LDH-Cytotoxicity Assay Kit provides a fast and simple method for quantitating necrosis based on the measurement of activity of lactate dehydrogenase (LDH) released from damaged cells. The formazan dye is water-soluble and can be detected by spectrophotometer at 500 nm.

Working Solutions

1. Reconstitute the Catalyst in 1 ml ddH$_2$O for 10 min and mix thoroughly. The Catalyst Solution is stable for several weeks at 4°C.
2. The Dye Solution is stable for several weeks at 4°C (and not at –20°C).
3. For 100 assays, mix 250 µl of Catalyst Solution with 11.25 ml of Dye Solution. The mixture solution should be prepared immediately before use.

The assay was done according to the following protocol:

4. Centrifuge the solution at 250 g for 10 min.

5. Background Control: Transfer 100 µl of unexposed preservation solution carefully into corresponding wells of an optically clear 96-well plate. The background value has to be subtracted from all other values.
6. Fresh Control: Transfer 100 µl of exposed fresh preservation solution into corresponding wells.
7. Test Sample: Transfer 100 µl of sample solution into corresponding wells.
8. Add 100 µl Reaction Mixture to each well and incubate for up to 30 min at room temperature. Protect the plate from light.
9. Measure the absorbance of all samples at 490-500 nm using a microtiter plate reader.

Islet Isolation

Islets were isolated from male Sprague Dawley rats. Briefly, rat pancreata were digested with collagenase (7.5 mg/pancreas in 30 mL HBSS and purified by Ficoll-density centrifugation. Hand-picked islets were washed twice with Hank's buffered saline solution before culture in medium-199 supplemented with 10% fetal bovine serum. Islets designated for static incubation were cultured for 2 hours before use.

The procedure was done according to the following protocol:

- Once removed from the animal, transfer the pancreases & spleen to 150 mm petridish containing a small amount of HBSS supplemented with 0.5% Penn/Strep. All work from this point on, should be carried out in the flow hood under sterile conditions.
- With a pair of non-toothed forceps and iris scissors, remove the spleen, and any fatty tissue that is on the pancreas. Discard the waste material.
- Once the pancreases are "clean", transfer them to a 50ml conical tube containing 10 mL of HBSS (4 pancreases per tube).

- With a pair of surgical scissors proceed to chop the pancreases (to ~2.5mm in size) in a fast and thorough manner.
- Once done chopping, fill the tube to the 50 mL mark and set aside, allowing time for the tissue to settle (~1 min).
- Remove the supernatant to the 15 mL mark, along with the fat and debris that collected at the top of the tube.
- Repeat the above steps (2nd - 6th) three times per tube. On the last chop, do not remove the supernatant, instead, fill the tube up to 50 mL and centrifuge at 1000 RPM, 1 min.
- Remove the supernatant, being careful not to disturb the pellet.
- Prepare collagenase solution: maximum of 4 pancreases/tube (1 mg of collagenase per 1 mL of HBSS). For every rat pancreas 7.5 mL of collagenase is required (30 mg collagenase per tube, along with 30 mL HBSS).
- Filter the collagenase solution (steriflip 0.22 um millipore), add the filtered solution to pancreas containing tubes, cap and wrap parafilm around the top of each tube.
- In a 37°C water bath, hand shake the tubes for 6-10 minutes, depending on how vigorous the shaking. Alternate the position of the tubes during the shaking period to ensure that there is even exposure of the tissue to the water bath.
- Once the collagenase digestion is complete, remove the parafilm and cap in the flowhood, wipe down the tubes with 70% Etanol.
- Divide each tube in half, immediately add cold HBSS (4°C) to bring the volume up to 50 mL. This stops the collagenase digestion.
- Centrifuge the tubes at 1000 RPM for 1 minute. Remove the supernatant.
- Resuspend the pellet in 15 mL of HBSS and vortex. Filter through a 500 μm filter (screen/filter apparatus). Rinse the tubes out 2 times (with ~5-10 mL of HBSS) and

then spray the screen (draw up 10 mL of HBSS into 10 cc syringe and then spray with 23 gauge needle).

- Bring volume up to 50 mL and centrifuge for 1 min at 1000 RPM.
- Remove all supernatant.
- Add 10 mL of the ficoll stock (25%) to each tube. Vortex once or twice, making sure that the pellet is entirely broken up.
- Slowly, while rotating the pipette around the inside edge of the tube, add 6 mL of the 23% ficoll. Taking caution not to mix this layer with the first (25%) one.
- Add 6 mL of each of the remaining gradients (20.5% and 11%).
- Cap and centrifuge the tubes at 2000 RPM for 20 minutes, making sure that the brake is turned off.
- Attach a 10 cc syringe to the cannula and keep inside a sterile 50 mL tube. While holding the ficoll/islet tubes up to the light, use the cannula to "pick" off the layer of islets that are found on the first interface.
- Place the drawn off islets into a 50 mL tube containing some HBSS (approximately 20 mL), placing no more than 25 mL of ficoll into each tube containing HBSS.
- Bring the volume to up to 50 mL, cap and centrifuge at 2000 RPM for 2 minutes.
- With vacuum, remove supernatant and repeat wash. Invert tubes to ensure that the pellet is broken up.
- Pour islets into sterile petri-dish, and rinse the original tube 2 more times (5 mL each rinse) with M199.
- Fill petridish with M199 supplemented with 0.5% Penn/Strep and 10% FBS.
- With a pasteur pipette connected to the pipette aide, count islets and place in a new (sterile) petridish containing supplemented M199, CMRL or Ham's F-10 media to culture (approximately 15 mls).
- Incubate at 37°C, 5% CO_2.

Islet Yield

Dithizone-stained samples were evaluated pre-and post-purification in duplicate to determine islet yield. Islets were counted and sized to normalize yields of islet equivalent (IE) to 150 µm as follows. A sample of what is seen under the microscope is given in Figure 2-2.

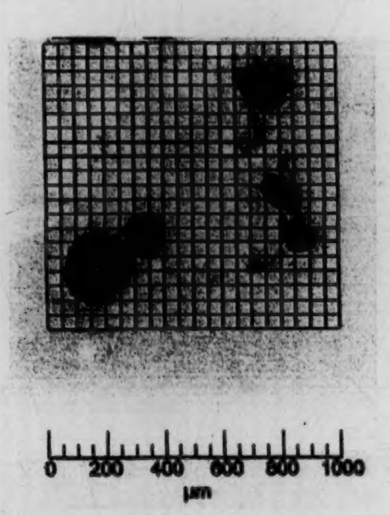
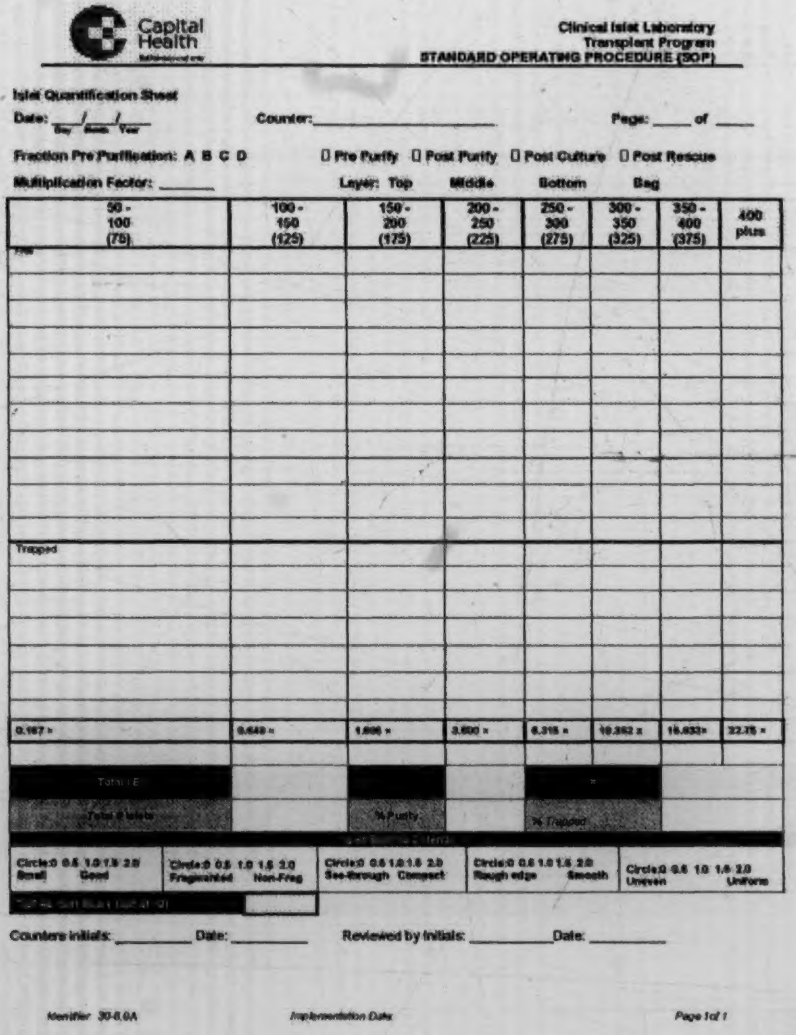


Figure 2-2: Schematic Islet count under the microscope

The counting was done according to the following protocol:

- Mix the final islet suspension very gently but thoroughly before taking a sample. Since islets settle rapidly in any container, care must be taken to properly resuspend the preparation in order to ensure a representative sample.
- Use a microdispensor pipette to quickly take a 100 ul sample from all layers separately or from final product and place the sample in a counting dish.
- Add a few drops of DTZ solution to the 100 ul sample in the counting dish and allow staining for a minimum of one minute. Count the islets under the microscope using the ×10 eyepiece and ×4 objective to give a total magnification of ×40. Use the certified grid in the eyepiece of the light microscope.

- The islets will appear red in a yellow background of exocrine tissue. DTZ stains the zinc in the insulin granules resulting in a characteristic red stain.



Capital Health

Clinical Islet Laboratory
Transplant Program
STANDARD OPERATING PROCEDURE (SOP)

Islet Quantification Sheet

Date: ___/___/___ (Day / Month / Year)   Counter: ____________________   Page: ____ of ____

Fraction Pre Purification: A B C D   ☐ Pre Purify ☐ Post Purify ☐ Post Culture ☐ Post Rescue

Multiplication Factor: ______   Layer: Top Middle Bottom Bag

50 - 100 (75)	100 - 150 (125)	150 - 200 (175)	200 - 250 (225)	250 - 300 (275)	300 - 350 (325)	350 - 400 (375)	400 plus
Trapped							
0.167 ×	0.648 ×	1.685 ×	3.500 ×	6.315 ×	10.352 x	15.833×	22.75 ×
Total IE				=			
Total # Islets		% Purity		% Trapped			

Islet Scoring Criteria

Circle: 0 0.5 1.0 1.5 2.0 Small Good	Circle: 0 0.5 1.0 1.5 2.0 Fragmented Non-Frag	Circle: 0 0.5 1.0 1.5 2.0 See-through Compact	Circle: 0 0.5 1.0 1.5 2.0 Rough edge Smooth	Circle: 0 0.5 1.0 1.5 2.0 Uneven Uniform

[illegible]

Counters initials: ________ Date: ________   Reviewed by initials: ________ Date: ________

Identifier 30-6.0A   *Implementation Date*   *Page 1of 1*

Figure 2-3: Islet Quantification Sheet

- Since islet volume is approximately proportional to the cube of the islet radius, it is crucial that the islets are divided into diameter (size) classes. Fifty um diameter range increments are used without considering particles smaller than 50 um since their contribution to the total volume of the preparation is insignificant. Quantify the islets as per following sheet.

- Islet Equivalent is calculated according to a standard chart (Figure 2-3).

Membrane Integrity Fluorescent Staining

Islet preparations were assessed for cell viability (ruling out the necrosis) using membrane exclusion dyes as described elsewhere [8]. A sample of islet preparations was stained using SYTO® Green (SYTO-13; Molecular Probes, Eugene, OR) and ethidium bromide. Following one minute incubation at room temperature, preparations were analyzed under fluorescent microscopy for proportion of live and dead cells, which then was expressed as a percentage (Figure 2-4).

Preparation of Stains:

- Thaw the SYTO stain at room temperature and centrifuge at 6000 rpm for 5 minutes before aliquoting, to pellet the DMSO.
- IN A DARKENED AREA: Dispense 1980 µL of DPBS into an eppendorf tube. Add 20 µL of SYTO, drawing liquid in and out of the pipette tip a few times to rinse.
- Aliquot the solution into 10X 200 µL volumes.
- Label and store, in the dark, at –20°C.
- IN A DARKENED AREA: Dispense 1980 µL of DPBS into an eppendorf tube. Add 20 µL of EB, drawing liquid in and out of the pipette tip a few times to rinse.
- Aliquot the solution into 20X 100µL volumes.
- Label and store, in the dark, at –20°C.
- Before use, allow the stains to warm to room temperature. Wrap the tubes in foil or keep them in a covered box because they are extremely light sensitive.

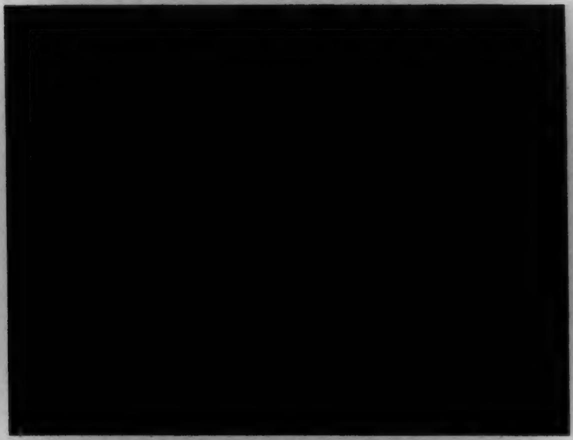


Figure 2-4: Islet viability with the fluorescence microscope

Sample Staining:

- Suspend an islet sample (100 µL) with the SYTO stain (10 µL).
- Allow the stain to set for 5 minutes.
- Add EB (5 µL) and lightly tap the eppendorf to suspend the islets.
- Allow the islet suspension to settle by gravity (~1min) and remove a 50 µL sample, drawing up from near the bottom of the tube.
- Dispense the sample along the length of a microscope slide in a vertical line and view.

Determination of Islet Viability:

- SYTO stains all cells green. Ethidium Bromide stains the cells red if the membrane is damaged.
- Record the number of cells that are viable or dead (If more than 50% of an islet is green, the islet is considered viable..
- Islets less than 50 µm are disregarded.
- Work quickly as the stain will be quenched rapidly.
- The stains will kill the cells over time, so they must be analyzed after the addition of EB.

- Once added to the slide, the sample will dry-out, so work quickly.

Islets Insulin Release Function

For assessment of *in vitro* insulin release function, islet preparations cultured at 37°C are examined for their secretory responsiveness to glucose during a static incubation. This assay is fast and reliable enough to show the viability of the islets. Perifusion method is a more accurate assay, but was not done as it is labor intensive and beyond the viability it shows ß-cell insulin synthesis ability.

Aliquots of 50 to 100 islets are incubated for 120 minutes in 1.5 mL of RPMI medium supplemented with 2 mM of L-glutamine, 0.5 % bovine serum albumin, and either 2.8 or 20 mM of glucose concentration. The supernatant is collected and frozen (-20°C) and subsequently assayed for insulin content by radioimmunoassay. Stimulation index (SI) is calculated by dividing insulin output during high-glucose (20 mM) incubation divided by insulin output during basal-glucose (2.8 mM) incubation.

Glucose solutions for static incubation assay

To prepare 1L **G-0 the following protocol was used**:

1. Add 900 mL of ddH_20 to a 1 L beaker
2. Stir while adding RPMI-1640 powder
3. Rinse bottle with ddH_20 to remove traces of powder
4. Decrease pH to 4.0 with 1N HCl (NB: this helps dissolve the proteins)
5. Increase pH to 7.2 with 1N NaOH
6. Add 2.0 g sodium biocarbonate per 1L final volume
7. Add 2.5 % Penn/Strep
8. Add 5 g Bovine Serum Albumin Fraction V powder (pour powder over approximately 800 ml of medium and allow to settle gradually versus quick stirring which will only form lumps and take longer to dissolve).
9. Adjust the pH to 7.2

To prepare 100 mL **G-439** (High glucose 20.0 mM), 439 mg D-glucose was dissolved in 100 mL of G-0 solution.

To prepare 100 mL **G-50** (Low glucose 2.8 mM),12.9 mL G-439 was dissolved in 100 mL G-0 solution.

To prepare a 2000 µM **IBMX stock** solution, 0.02222 g IBMX was dissolved in 50 mL G-0 solution, filtered and aliquoted into 10x5ml portions. Freeze at –20 °C.

To prepare 89 mL **G-439 + IBMX** (50 µM IBMX), 2.2 mL of IBMX stock was dissolved in 87 mL G-439 solution

The solutions were filtered with a 2 µm Bottle top filter and refrigerated at 4°C (3-week shelf life).

The assay was done according the following Protocol:

1. Label conical tubes and plates before starting (2 conical tubes/low glucose and 2/high).
2. Label tubes with type of islets, number of islets, incubation duration, date and low glucose or high glucose.
3. Wash plate: Transfer 6 mL of G-0 solution to a 6-well plate (3 wells per sample), label and incubate for 20 minutes at 37°C and 5% CO_2.
4. Treatment plate: In a labeled 6-well plate, pipet out one well of low glucose (6 mL) and one well of high glucose (6 mL) for every sample to be analyzed.
5. Incubate at 37°C and 5% CO_2 for at least 20 minutes.
6. Count out 50 islets and transfer into a cell strainer placed into a G-0 well (try to pick the same sized islets).
7. Incubate the plate (37°C and 5% CO_2) for at least 5 minutes.
8. Gently agitate the strainer then remove and transfer to another well for another wash.

9. Wash islets 3 times with 'No glucose' medium allowing them to sit for 5 minutes during each step.
10. After the final wash, transfer directly to the low glucose well and set the timer.
11. Repeat step 8 for each sample, recording the time for each sample.
12. Incubate at 37°C and 5% CO_2 for 60 minutes.
13. Remove the strainer and place into the high glucose well.
14. Remove the supernatant from the low glucose well (6 mL) and transfer to a 15 mL conical tube.
15. Allow the tube to sit for 1 minute.
16. Leave approximately 500 µL at the bottom in case some islets were accidentally lost through the strainer. Freeze the final tube (–20°C) immediately for the duration of the second incubation because insulin is unstable at lower temperatures.
17. Incubate the plate at 37°C and 5% CO_2 for an additional 60 minutes.
18. Once complete, repeat steps 12 to 14.
19. Transfer the final tubes to the -20°C freezer.
20. Samples stored at -20°C until sent for Enzyme-Linked ImmunoSorbent Assay (ELISA).

Statistics

All statistical analyses were performed using SPSS 11.05 software package SPSS, Inc., Chicago, IL). Metabolite data were presented as means ± SEM. Statistical differences between Groups were determined using one way analysis of variance (ANOVA) followed by [illegible] post-hoc test (Tukey's). A p-value of <0.05 was considered statistically significant.

Bibliography

[1] Atkinson DE. The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. *Biochemistry* 1968; 7: 4030.

[2] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351-358.

[3] Yagi K. Assay for blood plasma or serum. Methods Enzymol 1984; 105: 328-31.

[4] McCord JM. The evolution of free radicals and oxidative stress. Am J Med. 2000 Jun 1;108(8):652-9.

[5] Harward TR, Coe D, Souba WW, Klingman N, Seeger JM. Glutamine preserves gut glutathione levels during intestinal ischemia/reperfusion. J Surg Res 1994; 56: 351-5.

[6] Fernandez-Checa JC, Kaplowitz N. The use of monochlorobimane to determine hepatic GSH levels and synthesis. Anal Biochem 1990 Nov 1;190(2):212-9.

[7] Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J, Protein measurement with the Folin phenol reagent, J Biol Chem 1951; 193: 265-75.

[8] Barnett MJ, McGhee-Wilson D, Shapiro AMJ, Lakey JRT. Variation in human islet viability based on different membrane integrity stains. *Cell Transplant* 2004; 13: 481.

Chapter Three

Intraductal Oxygenation of Pancreas during Hypothermic Preservation

Introduction

Cold storage is applied to reduce acute tissue degeneration induced by warm ischemia; however, the main reason of cellular metabolic failure (hypoxia) remains unsolved and there is still a progressive deterioration of cellular survival over time. One avenue of studies is providing oxygen to pancreas during preservation. The two-layer cold storage method, University of Wisconsin (UW) solution and Perfluorocarbon (PFC), has been reported to continuously supply oxygen to pancreas during preservation and to reduce cold ischemic injury [1]. Canine pancreases subjected to 90 min of warm ischemia were resuscitated during preservation by the two-layer method (TLM) at 4°C for 24 to 48 hours [2]. One of the mechanisms of this method is to augment ATP, which maintains cellular integrity and controls ischemic cell swelling. The endogenous substrate for ATP synthesis is down regulated during ischemia. However, during the preservation by TLM, ATP is synthesized within the ischemically damaged pancreas by means of the direct phosphorylation of adenosine contained in the UW solution [3]. Recent investigations showed that the level of improvement in the quality and quantity of islets recovered by this method is not consistently superior to UW solution and the effects of TLM have been confounded by the huge variability between donor pancreases [4]. Since ATP is an essential source of energy to repair damaged cells, it is likely that ATP regeneration plays a key role in restoration of the ischemically damaged pancreas during preservation.

It has been reported, both in a rat and dog model, that islet yield improved from a stored pancreas after intraductal flush of collagenase resuspended in UW solution at the time of pancreas procurement [5]. Pre-cold storage injection of collagenase resuspended in buffer solution into the pancreatic duct of human pancreases from donors less than 30 years old also resulted in increased islet yields [6].

Islet isolation from a preserved pancreas is hampered by the exceptional susceptibility of the pancreatic ductal system to cold ischemic injury and improved preservation of the ductal epithelium would produce a better islet yield after pancreas preservation [7]. Therefore, it is appropriate to hypothesize that intraductal oxygenation may improve islet isolation outcome by increasing pancreatic tissue-UW solution contact and by preventing ischemic injury to ductal system. In this study, using intraductal injection of perfluorocarbon (PFC) was evaluated in rat model and changes in energy profiles were investigated.

Methods

Male Sprague-Dawley rats were used as donors (n=4, each group). Pancreas was procured according to standardized procurement procedures. Pancreata were removed after 20 ml intraductal injection of cold Hank's Balanced Salt Solution (HBSS), or pre-oxygenated solutions of HBSS (HBSSO), PFC (PFCO), and ePFC (ePFCO) and then preserved in HBSS for 24 hours. In all groups pre-oxygenation was done for 10 minutes with 100% oxygen at a flow rate of 2 L/min. Tissue samples were taken initially, 1, 4, 12 and 24 hours post-preservation; samples were snap-frozen in liquid nitrogen for later measurement of metabolism parameters and tissue protein contents. Spectrophotometric analysis was then performed to measure the absorbance of NADH at 340 nm, providing quantification of ATP, adenosine diphosphate (ADP), and adenosine monophosphate (AMP). Protein was measured according to Lowry et al. [8]. The energy charge (EC) was calculated according to Atkinson [9]: EC = (ATP + 0.5 ADP)/(ATP + ADP + AMP).

Results

ATP level (mean ± SEM) was significantly lower in all groups at 1, 4, 12, and 24 hours of preservation as compared to fresh (12.6 ± 1.6 μmol/g protein) (Figure 3-1). There was no significant difference in ATP values between HBSS, HBSSO, PFCO, and ePFCO groups at any time points.

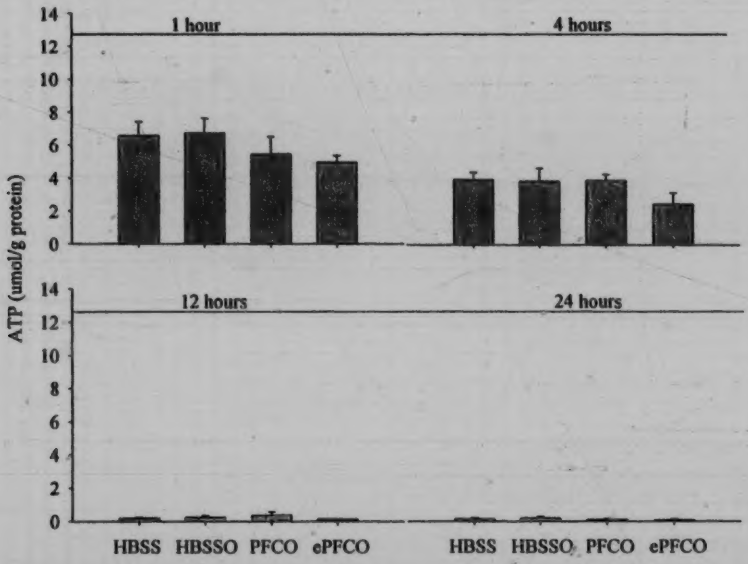


Figure 3-1: Adenosine tri-phosphate values of pancreatic tissues preserved in HBSS with intraductal injection of HBSS (HBSS), pre-oxygenated solutions of HBSS (HBSSO), PFC (PFCO), and ePFC (ePFCO). Line represents fresh value.

With exceptions in HBSS (10.8 ± 1.4 μmol/g protein) and HBSSO (10.8 ± 2.5 μmol/g protein) groups at 1 hour and PFCO (9.6 ± 2.9 μmol/g protein) group at 12 hours, total adenylate levels were significantly lower in all groups at any time points as compared to fresh (14.8 ± 2.1 μmol/g protein, $p<0.05$) (Figure 3-2). There was no significant difference in total adenylate values between groups at any time points of preservation.

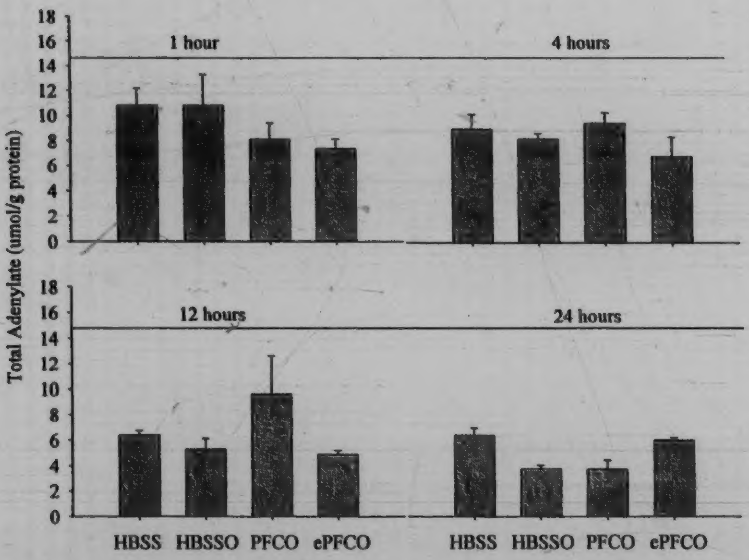


Figure 3-2: Total adenylate values of pancreatic tissues preserved in HBSS with intraductal injection of HBSS (HBSS), pre-oxygenated solutions of HBSS (HBSSO), PFC (PFCO), and ePFC (ePFCO). Line represents fresh value.

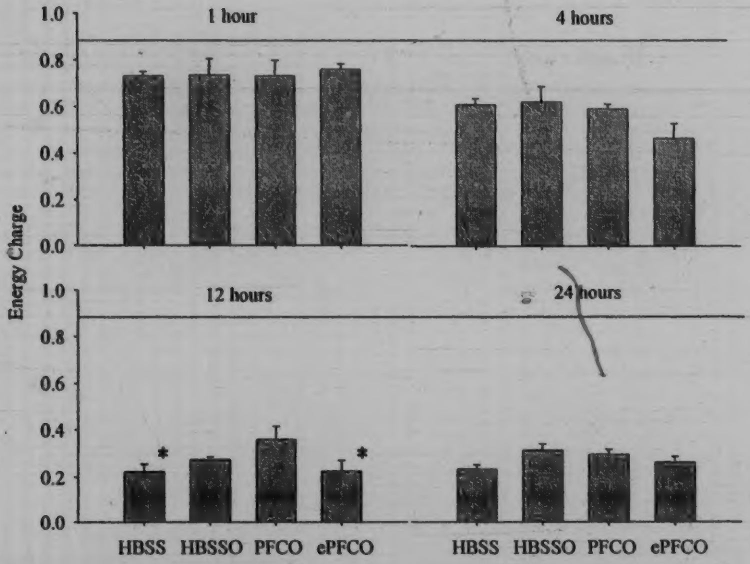


Figure 3-3: Energy charge values of pancreatic tissues preserved in HBSS with intraductal injection of HBSS (HBSS), pre-oxygenated solutions of HBSS (HBSSO), PFC (PFCO), and ePFC (ePFCO). Line represents the fresh value. * $p<0.04$ as compared to PFCO

Energy charge level was significantly lower in all groups as compared to fresh after 24 hours of preservation (Figure 3-3). There was no significant difference in energy charge values between groups at any time points except that significant higher value was seen in PFCO (0.36 ± 0.05) group at 12 hours of preservation as compared to HBSS (0.22 ± 0.03, P<0.04) group and ePFC (0.22 ± 0.04, P<0.04) group. ADP/ATP ratio level was significantly higher in all groups as compared to fresh after 1, 4, 12, and 24 hours of preservation (Figure 3-4). There was no significant difference in ADP/ATP ratio values between groups at 1, 4, 12, and 24 hours of preservation except that it was significantly lower in PFCO (8.57 ± 0.6, P<0.03) group as compared to HBSS (14.24 ± 2.4, P<0.03) group.

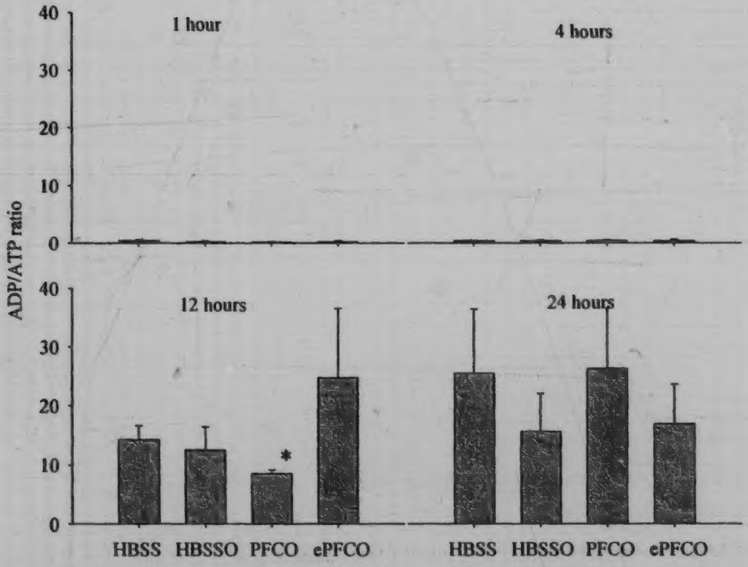


Figure 3-4: ADP-to-ATP ratio values of pancreatic tissues preserved in HBSS with intraductal injection of HBSS (HBSS), pre-oxygenated solutions of HBSS (HBSSO), PFC (PFCO), and ePFC (ePFCO). * p<0.03 as compared to HBSS group.

Discussion

In this study, Intraductal injection was shown to be not a suitable way to oxygenate the pancreas with the studied oxygen carriers as all groups showed significant lower values in energy profiles than fresh tissue. PFC improved energy charge as compared to the HBSS and ePFCO groups at 12 hours of preservation but the level of improvement was not significantly different as compared to the HBSSO groups. PFC could not improve the energy charge to over 50% which is considered as tissue viability cut point. It is believed that there are some potential beneficial effects of emulsified PFCs for ischemic tissue rescue and there are very strong indications that PFC emulsions will come to play an important part in the management of the ischemic tissues [10]; however in the present study intraductal injected ePFC could not improve energy profile as compared to the other studied groups.

There are reports available showing that intraductal injection of nutrients improves the islet isolation outcomes. Dr. Hering et al. showed that the islets isolated with their ductal preservation method (intraductal injection of UW solution alone in Lewis rat pancreas) functioned well, both in vitro (after overnight culture) and in vivo after transplantation [11]. The insulin release with ductal preservation was significantly higher than without ductal preservation, after both 6 and 24 hr of preservation. The primary advantage of their ductal preservation method is that it preserves the pancreatic duct, thereby improving the distribution of collagenase during post-cold storage ductal distention. The percentage of nonviable cells was significantly lower in the groups with ductal preservation than without ductal preservation, after both 6 and 24 hr of preservation. Avila et al. showed that intraductal glutamine administration at the time of enzyme perfusion considerably improves the islet yield, viability, and augments endogenous glutathione levels in pancreata procured after a clinically relevant period of ischemia [12].

Therefore, intraductal injection seems to be an appropriate way to provide nutrients for pancreas, but it's not suitable enough for oxygenating the tissue.

Not only pancreatic duct has been used for nutrient supply, but also used as digesting enzyme route. Munn et al. [5] reported that intraductal distention with collagenase resuspended in UW solution at the time of pancreas procurement improved islet yield in a rat and dog model. Their findings were subsequently confirmed by Ohzato et al. [13]. White et al. reported that islet yield markedly improved by intraductal collagenase distention, immediately after procurement and before preservation, in a porcine pancreas model [14]. Intraductal collagenase distention before pancreas preservation seems to be beneficial in islet isolation [6]. However, Casanova et al. [15] reported that intraductal collagenase distention in UW solution before pancreas storage for 14 to 16 hr produced inferior islet yield, fragmented the big- and medium-sized islets, and caused low-grade digestion of pancreases. It is generally believed that intraductal distention with collagenase in UW solution, at procurement, cannot be used in islet isolation. However, the reason is not the efficiency of intraductal way, it's due to the inhibitory effect of components of UW solution on collagenase during the preservation of the pancreas [16].

Taken together, intraductal injection of PFC as an alternative method of increasing tissue oxygenation during rat pancreas preservation improved energy charge at 12 hours of preservation, however, this method displayed dramatic inferior energy profile as compared to fresh biopsies and comparable to that of bubbling of oxygen group.

Bibliography

---

[1] Kuroda Y, Fujino Y, Morita A, Tanioka Y, Ku Y, Saitoh Y. Oxygenation of the human pancreas during preservation by a two-layer (University of Wisconsin solution/perfluorochemical) cold-storage method. Transplantation. 1992; 54(3):561-2.

[2] Kuroda Y, Tanioka Y, Morita A, Hiraoka K, Matsumoto S, Fujino Y, Ku Y, Saitoh Y, Sugihara J, Okumura S, et al. The possibility of restoration of human pancreas function during preservation by the two-layer (University of Wisconsin solution/perfluorochemical) method following normothermic ischemia. Transplantation. 1994; 57(2):282-5.

[3] Kuroda Y, Hiraoka K, Tanioka Y, Matsumoto S, Morita A, Fujino Y, Suzuki Y, Ku Y, Saitoh Y. Role of adenosine in preservation by the two-layer method of ischemically damaged canine pancreas. Transplantation. 1994; 57(7):1017-20.

[4] Hering BJ, Matsumoto I, Sawada T, Nakano M, Sakai T, Kandaswamy R, Sutherland DE. Impact of two-layer pancreas preservation on islet isolation and transplantation. Transplantation. 2002 Dec 27;74(12):1813-6

[5] Munn SR, Kaufman DB, Field MJ, et al. Cold-storage preservation of the canine and rat pancreas prior to islet isolation. Transplantation 1989; 47: 28.

[6] Socci C, Davalli AM, Vignali A, et al. A significant increase of islet yield by early injection of collagenase into the pancreas duct of young donors.

[7] Sawada T, Matsumoto I, Nakano M, Kirchhof N, Sutherland DE, Hering BJ. Improved islet yield and function with ductal injection of University of Wisconsin solution before pancreas preservation. Transplantation. 2003 Jun 27;75(12):1965-9

[8] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265

[9] Atkinson DE. The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. Biochemistry 1968; 7: 4030

[10] Lowe KC. Perfluorochemical oxygen carriers and ischaemic tissues. Adv Exp Med Biol. 1990;277:257-66.

[11] Sawada T, Matsumoto I, Nakano M, Kirchhof N, Sutherland DE, Hering BJ. Improved islet yield and function with ductal injection of University of Wisconsin solution before pancreas preservation. Transplantation. 2003 Jun 27;75(12):1965-9.

[12] Avila JG, Tsujimura T, Oberholzer J, Churchill T, Salehi P, Shapiro AM, Lakey JR. Improvement of pancreatic islet isolation outcomes using glutamine perfusion during isolation procedure. Cell Transplant. 2003;12(8):877-81.

[13] Ohzato H, Gotoh M, Monden M, et al. Improvement in islet yield from a cold-preserved pancreas by pancreatic ductal collagenase distention at the time of harvesting. Transplantation 1991; 51: 566.

[14] White SA, Hughes DP, Contractor HH, et al. The intraductal administration of collagenase in University of Wisconsin solution at the time of harvesting significantly improves porcine islet isolation. Transplant Proc 1995; 27: 3366.

---

[15] Casanova D, Xenos E, Lloveras JJ, et al. Comparison of human islet isolation from the stored and nonstored pancreas with two different protocols using UW solution. Transplant Proc 1994; 26: 588.

[16] Contractor HH, Johnson PR, Chadwick DR, et al. The effect of UW solution and its components on the collagenase digestion of human and porcine pancreas. Cell Transplant 1995; 4: 615.

Chapter Four

Continuous Oxygenation of Pancreas during Hypothermic Preservation

Introduction

Oxygen is necessary for cellular processes, most notably the production of ATP by cellular respiration through oxidative phosphorylation [1]. There are several reports of oxygenating tissues with different means during the past several decades. It has been documented that the provision of sufficient oxygen by retrograde oxygen persufflation can restore viability of ischemically damaged kidneys [2] and livers [3]. The viability of rat livers harvested from non-heart-beating donors with 30 to 60 minutes of warm ischemia was improved by subsequent treatment with oxygen persufflation during 24-hour cold storage [4]. It has been demonstrated that oxygenation of a pancreas during preservation using a two layer method (TLM) improves ATP production and maintains a superior integrity and viability of islets as compared to University of Wisconsin (UW) solution [5]. It has been reported that the pancreas is not oxygenated during preservation in UW with continuous oxygen bubbling [6]. In this study, tissue oxygen tension (PO_2) of the ischemically damaged pancreas graft was measured directly during preservation either by the two-layer (UW/PFC) method or simple storage in UW with oxygen bubbling at 20°C.

Over the years, three types of oxygenators have been constructed and used clinically: Bubble-type oxygenator; Membrane-type; and Film-type oxygenator. In bubble oxygenators, gas is introduced into the blood directly in the form of bubbles. The oxygenation takes place effectively because of the large surface area of the bubbles and so it is one of the most effective and simplest oxygenators. Because of the mechanical stress induced by the introduction of air bubbles into the blood and also because of the direct contact of air bubbles with the blood, the trauma caused by this means of oxygenation is the highest of all oxygenators. Even though blood trauma induced by this oxygenator is high, it was widely used for short-duration bypass procedures because it is not only inexpensive but also easy to use [7]. In this study, the potential of continuous oxygen bubbling method in improving pancreatic energy profile is evaluated in rat pancreases with prolonged cold storage in UW solution.

Methods

Pancreas was procured from male Sprague-Dawley rats (n=4, each group) according to standardized procurement procedures. Pancreata were removed after 20 ml intraductal injection of cold Hank's Balanced Salt Solution (HBSS), and preserved in HBSS for 24 hours. In the group with continuous bubbling of oxygen, pre-oxygenation was done for 10 minutes with 100% oxygen at a flow rate of 2 L/min and continuous 100% oxygen bubbling in a flow rate of 0.2 L/min during 24 hours of preservation. Tissue samples were taken initially and at 1, 4, 12, and 24 hours post-procurement; samples were snap-frozen in liquid nitrogen for later measurement of metabolism parameters and tissue protein contents. Spectrophotometric analysis was then performed to measure the absorbance of NADH at 340 nm, providing quantification of ATP, adenosine diphosphate (ADP), and adenosine monophosphate (AMP). Protein was measured according to Lowry et al. [8]. The energy charge (EC) was calculated according to Atkinson [9]: EC = (ATP + 0.5 ADP)/(ATP + ADP + AMP).

Results

ATP level (mean ± SEM) was significantly lower in both groups as compared to fresh after 1, 4, and 12 hours of preservation, however there was no significant difference in ATP value between oxygen bubbling group (12.2 ± 0.7 µmol/g protein) and fresh (12.8 ± 0.9 µmol/g protein, NS) after 24 hours of preservation.

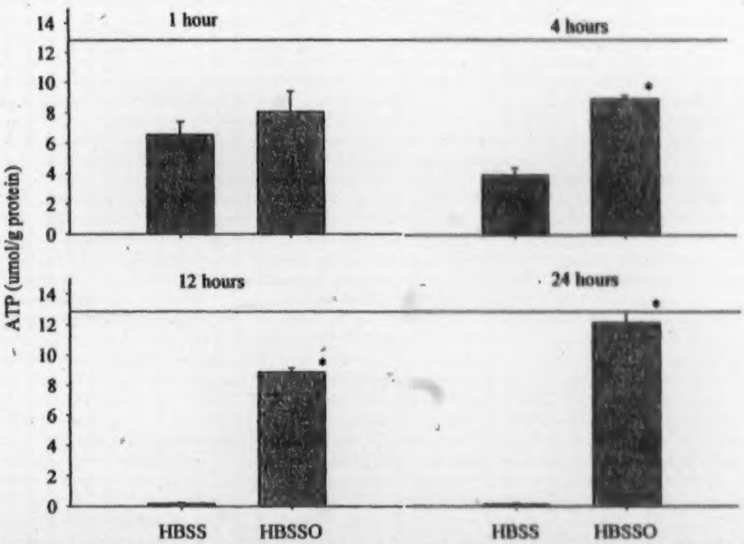


Figure 4-1: ATP content of pancreatic tissue during 24 hours of cold preservation in HBSS with & without continuous bubbling of oxygen. *p<0.01 as compared to HBSS group. Line represents fresh value.

After 4 hours of preservation, ATP level was significantly and constantly higher in oxygen bubbling group (9.0 ± 0.2 µmol/g protein) as compared to HBSS (3.9 ± 0.4 µmol/g protein, p<0.001) group (Figure 4-1). Total adenylates levels were significantly lower in both groups as compared to fresh after 4, and 12 hours of preservation, however there were no significant differences in total adenylates values between oxygen bubbling group and fresh after 1 and 24 hours of preservation (Figure 4-2). At 4 hours of preservation, total adenylates level was significantly and constantly higher in oxygen bubbling (12.4 ± 0.5 µmol/g protein) group as compared to HBSS (9.0 ± 1.1 µmol/g protein, p<0.01) group. Energy charge level was significantly lower in both groups as compared to fresh after 1, 4, and 12 hours of preservation, however there was no significant difference in energy charge value between oxygen bubbling group and fresh after 24 hours of preservation (Figure 4-3). At 4 hours of preservation, Energy charge level was significantly and constantly higher in oxygen bubbling (0.84 ± 0.009) group as compared to HBSS (0.61 ± 0.02, $p<0.001$) group and afterwards. Energy charge remained in the normal range after 24 hours of pareservation.

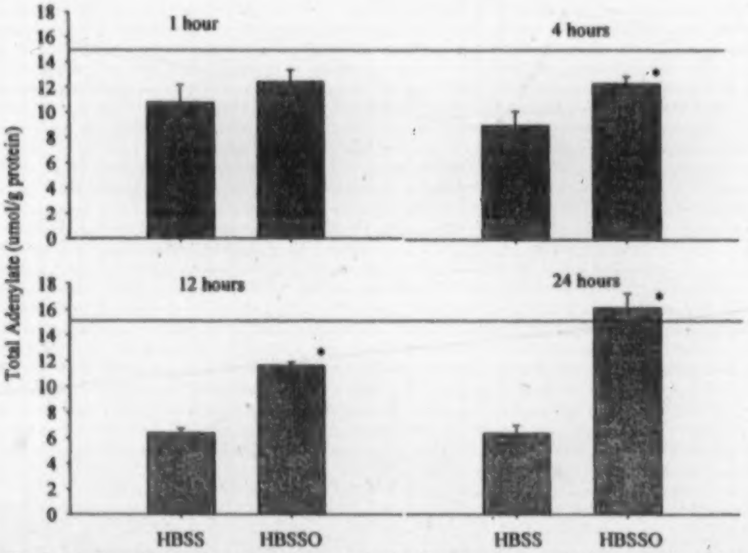


Figure 4-2: Toatal adenylates content of pancreatic tissue during 24 hours of cold preservation in HBSS with & without continuous bubbling of oxygen. *$p<0.001$ as compared to HBSS group. Line represents fresh value.

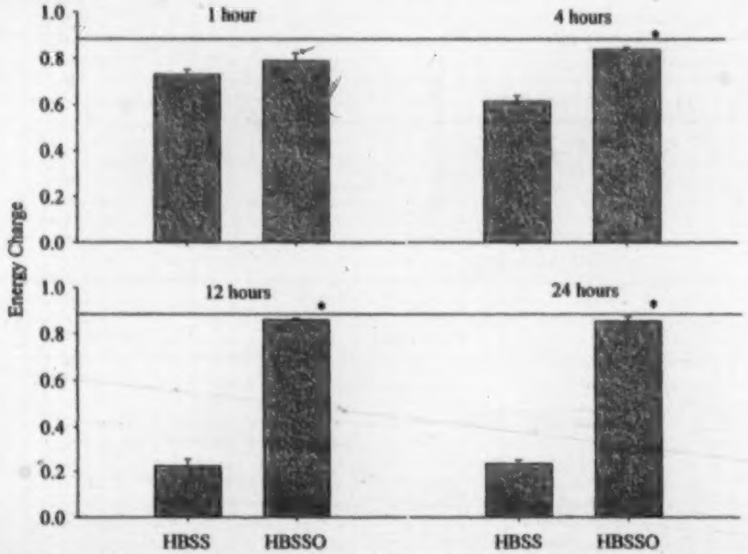


Figure 4-3: Energy charge content of pancreatic tissue during 24 hours of cold preservation in HBSS with & without continuous bubbling of oxygen. *$p<0.001$ as compared to HBSS group. Line represents fresh value.

ADP/ATP ratio was significantly higher in both groups as compared to fresh after 1, 4, and 12 hours of preservation, however there was no significant difference in ADP/ATP ratio value between oxygen bubbling group and fresh after 24 hours of preservation (Figure 4-4). At 4 hours of preservation, ADP/ATP ratio level was significantly and constantly lower in oxygen bubbling (0.30 ± 0.02) group as compared to the HBSS (0.76 ± 0.11, p<0.001) group, and afterwards.

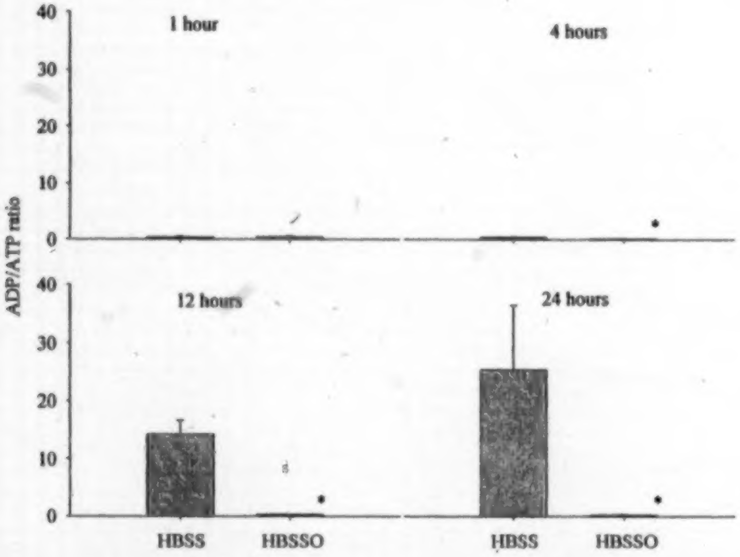


Figure 4-4: ADP/ATP ratio of pancreatic tissue during 24 hours of cold preservation in HBSS with & without continuous bubbling of oxygen. *p<0.001 as compared to HBSS group.

Discussion

In the present study, continuous bubbling of oxygen in the preservation solution could improve the pancreatic tissue energy profile as compared to non-oxygenated group. It improved energy profile significantly at 4 hours of pancreas preservation and afterwards. The energy profile reached to those of fresh values at 24 hours which is a promising result. ATP has two different roles in the cells. Firstly, it is a source of energy that keeps the cells alive. Oxidative phosphorylation pathway through mitochondria is the main route of producing ATP within the cells which is probably the mechanisms involved in reversing ATP supply to normal levels. Secondly, it is a regulator of insulin secretion in beta cells. Not only does elevated ATP in the experimental group imply improved viability, significantly lower ADP/ATP ratio (an index of apoptosis) [10] may implicate a reduction in apoptotic signals. Tanioka et al. showed that with augmentation of tissue ATP level during digestion results in high islet yield [11] and Sweet et al. showed that ATP and ADP levels are critical regulators of glucose stimulated insulin secretion in islets [12].

Measuring of tissue oxygen tension (PO_2) of the ischemically damaged pancreas graft directly during preservation either by the two-layer (UW/PFC) method or simple storage in UW with oxygen bubbling at 20°C has shown that the pancreas is not oxygenated during preservation in UW with continuous oxygen bubbling [13]. However, in another organ like small intestine, there is a report demonstrates that without oxygen bubbling, the two-layer method (TLM) is not effective for long time small bowel preservation [14]. Lakey et al. showed that 16 hours was the upper limit for cold storage of human pancreases before islet isolation [15] and greater than 8 hours of cold storage before human islet isolation significantly reduced islet yield and purity [16]. This is for the first time ever that the pancreatic tissue ATP content could reach to fresh values after 24 hours of preservation in this study. Based on these data, I hypothesize that continuous bubling of oxygen could prevent islet death during pancreas preservation and improve islet isolation outcome. In the next chapter the effects of oxygen bubbling will be compared to TLM. The mechanisms involved and the islet isolation outcome will be evaluated.

Bibliography

[1] Brunelle JK, Chandel NS. Oxygen deprivation induced cell death: an update.Apoptosis. 2002 Dec;7(6):475-82.

[2] Rolles K, Foreman J, Pegg DE. Preservation of ischemically injured canine kidneys by retrograde oxygen persufflation. Transplantation 1984; 38: 102.

[3] Minor T, Klauke H, Isselhard W. Resuscitation of cadaveric livers from non-heart beating donors after warm ischemic insult: a novel technique tested in the rat. Experientia 1996; 52: 661.

[4] Minor T, Saad S, Kitting M, et al. Short term restoration by gaseous oxygen for long term preservation of predamaged livers from non heart beating donors. Transplant Proc 1997; 29: 3474.

[5] Hiraoka K, Kuroda Y, Tanioka Y et al. The importance of adenosine and a colloid (Dextran 40) in resuscitation of ischemically damaged canine pancreas during preservation by the two-layer method. Transplantation 1994; 58: 1279-1281.

[6] Matsumoto S, Kuroda Y, Hamano M, Kim Y, Suzuki Y, Ku Y, Saitoh Y. Direct evidence of pancreatic tissue oxygenation during preservation by the two-layer method. Transplantation. 1996 Dec 15;62(11):1667-70.

[7] Iwahashi H, Yuri K, Nose Y. Development of the oxygenator: past, present, and future.J Artif Organs. 2004;7(3):111-20

[8] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265

[9] Atkinson DE. The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. Biochemistry 1968; 7: 4030

[10] Bradbury DA, Simmons TD, Slater KJ, Crouch SP. Measurement of the ADP:ATP ratio in human leukaemic cell lines can be used as an indicator of cell viability, necrosis and apoptosis. J Immunol Methods. 2000 Jun 23;240(1-2):79-92

[11] Tanioka Y, Tanaka T, Gotoh T, Kakinoki K, Li S, Yoshikawa T, Sakai T, Fujino Y, Suzuki Y, Kuroda Y. Augmentation of tissue ATP level during digestion using preoxygenated perfluorocarbon results in high islet yield-new strategy for islet isolation. Transplant Proc 2005; 37:220

[12] Sweet IR, Cook DL, DeJulio E, Wallen AR, Khalil G, Callis J, Reems J.Regulation of ATP/ADP in pancreatic islets. Diabetes 2004; 53:401.

[13] Matsumoto S, Kuroda Y, Hamano M, Kim Y, Suzuki Y, Ku Y, Saitoh Y. Direct evidence of pancreatic tissue oxygenation during preservation by the two-layer method. Transplantation. 1996 Dec 15;62(11):1667-70.

[14] Kuroda Y, Sakai T, Suzuki Y, Tanioka Y, Matsumoto S, Kim Y, Fujita H, Hamano M, Hasegawa Y, Ku Y, Saitoh Y. Small bowel preservation using a cavitary two-layer (University of Wisconsin solution/perfluorochemical) cold storage method. Transplantation. 1996 Feb 15;61(3):370-3.

---

[15] Lakey JR, Rajotte RV, Warnock GL, et al. Human pancreas preservation prior to islet isolation. Transplantation 1995; 59: 689.

[16] Zeng Y, Torre MA, Karrison T, et al. The correlation between donor characteristics and the success of human islet isolation. Transplantation 1994; 57: 954.

Chapter Five

Continuous Bubbling of Oxygen during Pancreas Preservation: Effects and Mechanisms

Introduction

During pancreas procurement, blood supply and hence oxygen is necessarily interrupted. Although cold storage is applied to reduce tissue degeneration, there is a progressive deterioration of cellular function over time. During ischemia, mismatch balance between supply and demand of adenosine triphosphate (ATP) eventually results in mitochondrial oxidative phosphorylation suppression followed by necrosis and apoptosis [1]. Apoptosis observed in islets has been reported mainly to be mitochondria-dependent, mediated by change in redox potential initiated by hypoxia [2]. It has been demonstrated that oxygenation of a pancreas during preservation using a two layer method (TLM) improves ATP production and maintains a superior integrity and viability of islets as compared to University of Wisconsin (UW) solution [3].Brandhorst et al. even reported that omitting the University of Wisconsin (UW)-layer could accelerate oxygen release into the ambient atmosphere by measurement of oxygen tension in PFC pre-charged for One-Layer-Method as compared to that for TLM [4]. However, the level of improvement in the quality and quantity of islets recovered by this method is not consistently superior to UW solution and the effects of TLM have been confounded by the huge variability between donor pancreases [5].

It has been reported that the pancreas is not oxygenated during preservation in UW with continuous oxygen bubbling [6]. Tissue oxygen tension (PO_2) of the ischemically damaged pancreas graft was measured directly during preservation either by the two-layer (UW/PFC) method or simple storage in UW with oxygen bubbling at 20°C. The main confounding factor with the study is that the oxygen partial pressure in UW solution decreases with the increasing of temperature and 20°C is not a temperature currently used for preservation. Years later, the same group showed that there is no difference between pre-oxygenated TLM (static TLM) and the original TLM with continuous oxygen supply [7]. In contrast, there is another report demonstrates that without oxygen bubbling, the two-layer method is not effective for long time small bowel preservation [8]. In the latter study, survival rate of the transplanted graft was assessed by graft necrosis through autopsies 7 days after transplantation.

In chapter 4, the amount of oxygen dissolved in the HBSS solution by continuous bubbling was shown to be suitable enough to improve energetic content of pancreatic tissue during 24 hours of preservation. In this chapter, continuous bubbling of oxygen in UW solution is compared with TLM in terms of reducing hypoxia of rat preserved pancreas and providing protection to pancreatic islets during pancreas preservation. Mechanisms involved in cellular death (necrosis and apoptosis: intrinsic and extrinsic pathways) during preservation time and their effects on islet isolation outcomes are studied.

Methods

Male Sprague-Dawley rats were used as donors (n=4 in each group). Pancreas was procured according to standardized procurement procedures. Pancreata were removed after 20 ml of intraductal injection of filtered UW, and preserved for 24 hours in four different groups including: UW solution, TLM, UW with continuous bubbling of oxygen (UWO), and TLM with continuous bubbling of oxygen (TLMO). Pre-oxygenation was done for 10 minutes with 100% oxygen at a flow rate of 2 L/min and continuous 100% oxygen bubbling in a flow rate of 0.2 L/min during 24 hours of preservation. Since PFC has a high capacity of Co_2 solubility, 100% oxygen was employed to eliminate this confounding factor. Tissue samples (100 mg) were collected at 0, 1, 4, 12, and 24 hours after procurement. To arrest metabolic activity, samples were snap-frozen in liquid nitrogen and subsequently stored at -80 °C until processed. Aliquots of neutralized extracts were processed via standard enzyme-linked metabolite assays [9].

Spectrophotometric analysis was then performed to measure the absorbance of NADH at 340 nm, providing quantification of ATP, ADP, AMP, and lactate. We quantitatively measured Reduced Glutathatione (GSH) using monochlorobimane [10] and malondialdehyde (MDA) according to Ohkawa [11]. DNA fragmentation was measured based on the quantitative "sandwich enzyme immunoassay" principle using mouse monoclonal antibodies directed against DNA and histones [12]. The caspase 3, 8, and 9 colorimetric assays were based on the hydrolysis of the peptide substrate by caspases, resulting in the release of the p-nitroaniline (pNA) moiety [13]. To standardize metabolite data, values were reported in terms of 'per gram protein'. Protein was measured according to Lowry et al [14]. Biopsies were stained for structure (Hematoxylin & Eosin), for β-cells (insulin-positive), and for α-cells (glucagon-positive) by an immunoperoxidase technique.

Solution's pH was measured by symphony SB21 pH meter (VWR Scientific products). The Machine was calibrated to measure pH at 4°C. Necrosis was quantified based on the measurement of activity of lactate dehydrogenase (LDH) released from damaged cells. The partial oxygen pressure in UW and PFC was measured using a Foxy fiber optic oxygen sensor (Ocean Optics, Inc., FL).

Rat pancreata were digested with collagenase and purified by Ficoll-density centrifugation. Dithizone-stained samples were evaluated post-purification in duplicate to determine islet yield. Islets were counted and sized to normalize yields of islet equivalent (IEQ) to 150 µm. A sample of islet preparations was stained using SYTO Green (SYTO-13; Molecular Probes, Eugene, OR) and ethidium bromide. For assessment of in vitro function, islet preparations cultured at 37°C were examined for their secretory responsiveness to glucose during a static incubation. Metabolite data were presented as means ± SEM. Statistical differences between Groups were determined using ANOVA followed by a post-hoc test (Tukey's).

Results

Oxygen Partial pressure in UW Solution

Oxygen partial pressure in UW solution was significantly higher in UWO group (18.35±1.48%) as compared to UW (3.65±0.45%, $p<0.001$) and TLM groups (8.12±0.38 %, $p<0.001$) at 1 hour of preservation and remained so during the whole 24 hours of pancreas preservation (Figure 5-1). Oxygen partial pressure in UW solution of TLM group was significantly higher than that of UW group but significantly lower than that of UWO group at 1 hour of preservation and remained so during the whole 24 hours of pancreas preservation. In spite of high dissolved oxygen levels in the PFC layers, oxygen partial pressure in the UW solutions in both TLM and TLMO groups were significantly lower than those of PFCs. TLMO group did not show any significant difference in UW dissolved oxygen content as compared to UWO group.

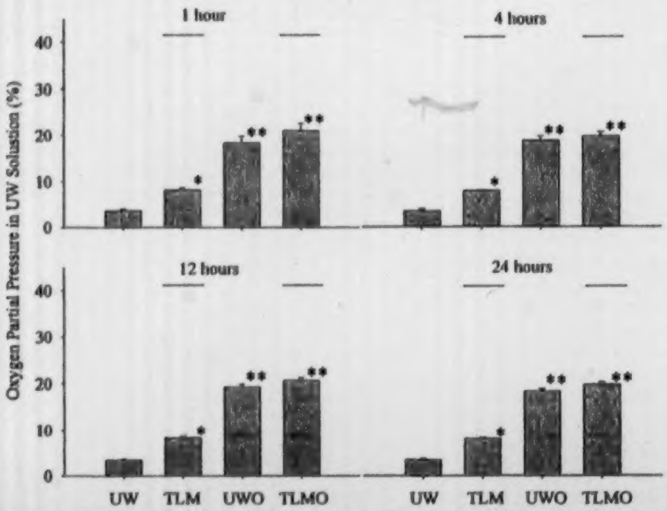


Figure 5-1: Oxygen partial pressure in UW solution (%) during 24 h pancreas preservation. UW: University of Wisconsin solution, TLM: Two-Layer Method, UWO: UW with continuous bubbling of oxygen, TLMO: TLM with continuous bubbling of oxygen. Lines represent oxygen partial pressure in the PFC layer of TLM. * $p<0.001$ as compared to UW group; ** $p<0.001$ as compared to UW and TLM group

Intracellular Energy Profile

ATP level of pancreatic tissue was significantly higher in UWO group (9.43±0.81 umol/g protein) as compared to UW (7.40±0.21 umol/g protein, p<0.001) and TLM groups (7.10±0.69 umol/g protein, p<0.001) at 1 hour of pancreas preservation and remained so afterwards (Figure 5-2). ATP level of pancreatic tissue was significantly higher in TLM group than that of UW group but significantly lower than that of UWO group at 12 and 24 hours of preservation.

There was no significant difference in total adenylates levels between the groups and as compared to fresh procured tissue during the whole preservation time (Figure 5-3). The only exception is that total adenylates level in UW group at 4 hours was significantly higher than that of fresh procured tissue (25.96±2.06 vs. 13.50±0.91 umol/g protein, p<0.001).

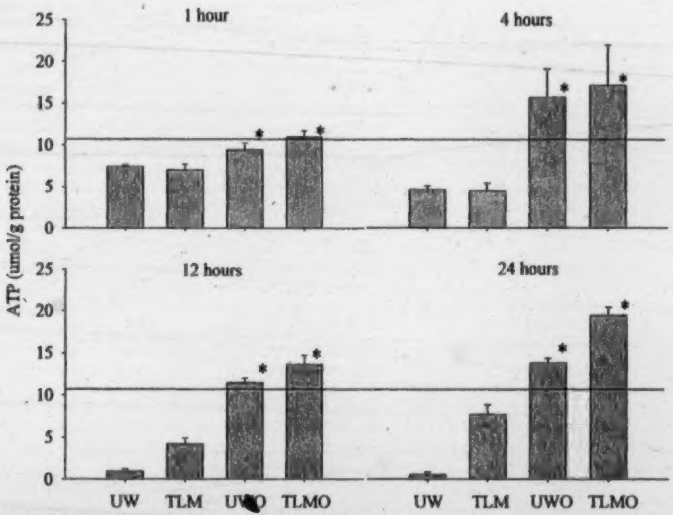


Figure 5-2: ATP level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, TLM: Two-Layer Method, UWO: UW with continuous bubbling of oxygen, TLMO: TLM with continuous bubbling of oxygen. Line represents the fresh value. *p<0.01 as compared to UW group

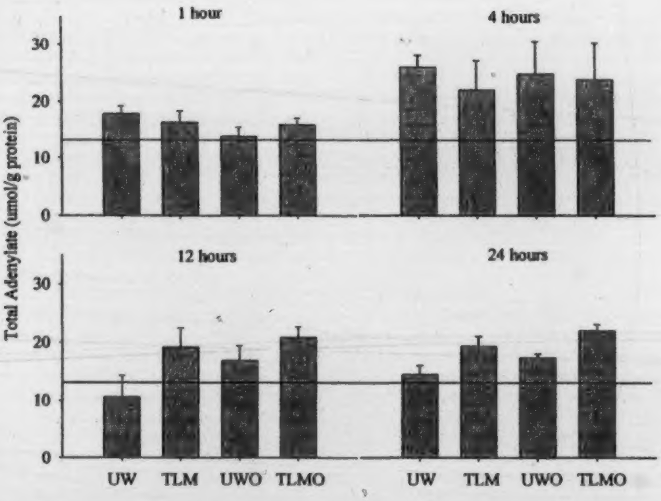


Figure 5-3: Total adenylates of pancreatic tissue during 24 h pancreas preservation. UW: University of Wisconsin solution, TLM: Two-Layer Method, UWO: UW with continuous bubbling of oxygen, TLMO: TLM with continuous bubbling of oxygen. Line represents the fresh value.

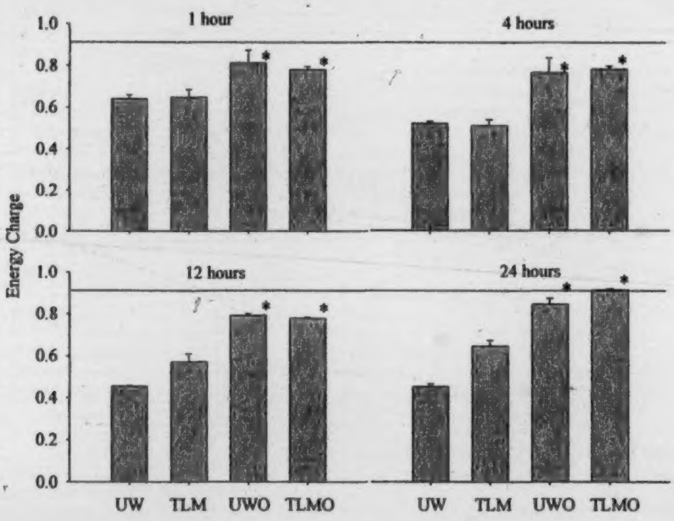


Figure 5-4: Energy charge level of pancreatic tissue during 24 h pancreas preservation. UW: University of Wisconsin solution, TLM: Two-Layer Method, UWO: UW with continuous bubbling of oxygen, TLMO: TLM with continuous bubbling of oxygen.Line represents the fresh value. *p<0.001 as compared to UW group

Energy charge (EC) level of pancreatic tissue was significantly higher in UWO group (0.81±0.06) as compared to UW (0.64±0.02, p<0.001) and TLM groups

(0.65±0.04, $p<0.001$) at 1 hour of pancreas preservation and afterwards (Figure 5-4). EC level of pancreatic tissue was significantly higher in TLM group than that of UW group but significantly lower than that of UWO group at 12 and 24 hours of preservation. Continuous bubbling of oxygen in TLMO group showed no significant difference in EC level as compared to continuous bubbling of oxygen in UWO group during 24 hours of pancreas preservation. In both UWO and TLMO groups, there was no significant difference in EC levels as compared to fresh tissue at 24 h of pancreas preservation.

ADP/ATP ratio level of pancreatic tissue was significantly lower in UWO group (0.38±0.28) as compared to UW (1.05±0.10, $p<0.001$) and TLM groups (0.99±0.16, $p<0.001$) at 1 hour of pancreas preservation and afterwards. Continuous bubbling of oxygen in TLMO group showed no significant difference in ADP/ATP ratio level as compared to continuous bubbling of oxygen in UWO group during 24 hours of pancreas preservation (Figure 5-5).

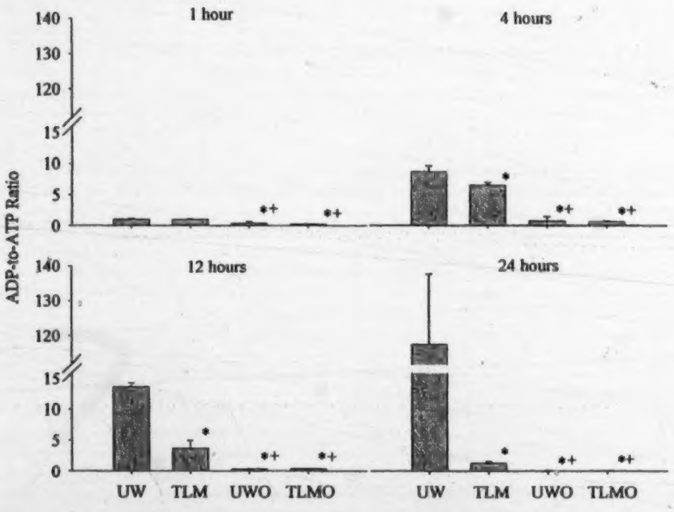


Figure 5-5: ADP/ATP ratio level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, TLM: Two-Layer Method, UWO: UW with continuous bubbling of oxygen, TLMO: TLM with continuous bubbling of oxygen. * $p<0.03$ as compared to UW group; + $p<0.001$ as compared to TLM group.

In both UWO and TLMO groups, there was no significant difference in ADP/ATP ratio levels as compared to fresh procured tissue at 24 hours of pancreas preservation.

Anaerobic metabolism

Lactate level of pancreatic tissue was significantly lower in UWO group (3.19±0.49 umol/g protein) as compared to UW (9.06±0.37 umol/g protein, $p<0.001$) and TLM groups (7.93±1.74, umol/g protein $p<0.001$) at 4 hours of pancreas preservation and afterwards (Figure 5-6). TLM group also showed a significant decrease in lactate level as compared to UW group at 1 hour of pancreas preservation, bur not remained significant afterwards. In UWO group, there was a significant decrease in lactate levels as compared to fresh procured tissue at 12 and 24 hours of pancreas preservation. In both UWO and TLMO groups, there was a significant increase in UW solution's pH levels as compared to those of fresh, UW and TLM groups (Figure 5-7).

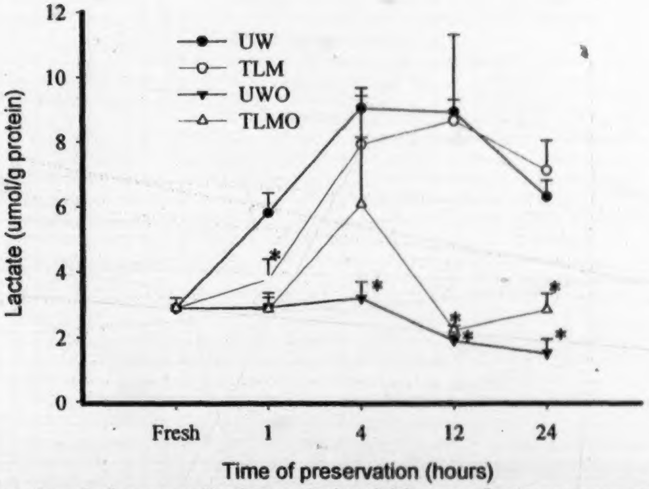


Figure 5-6: Lactate level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, TLM: Two-Layer Method, UWO: UW with continuous bubbling of oxygen, TLMO: TLM with continuous bubbling of oxygen.* $p<0.001$ as compared to UW group

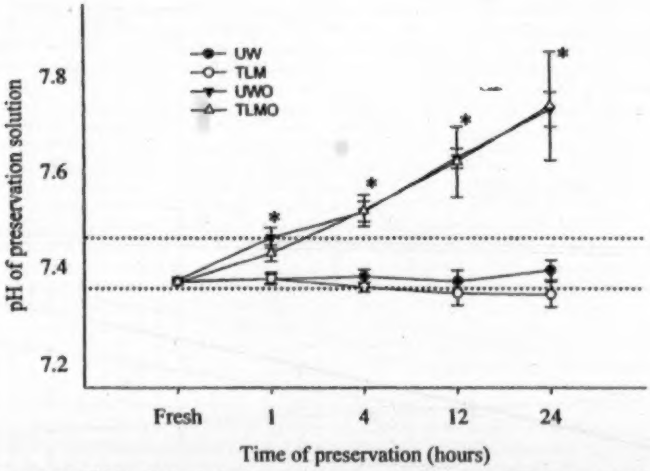


Figure 5-7: pH level of UW solution during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, TLM: Two-Layer Method, UWO: UW with continuous bubbling of oxygen, TLMO: TLM with continuous bubbling of oxygen. * $p<0.001$ as compared to UW and TLM groups

Oxidative Stress

GSH level of pancreatic tissue was significantly lower in UWO group (1.59±0.36 umol/g protein) as compared to UW (3.51±0.27 umol/g protein, $p<0.001$) and TLM groups (4.06±1.04 umol/g protein, $p<0.05$) at 24 hours of pancreas preservation (Figure 5-8). There was no significant difference in GSH levels in TLM group as compared to UW group during 24 hours of pancreas preservation. There was also no significant difference in MDA levels between all groups during 24 hours of pancreas preservation and no significant increase as compared to fresh pancreatic tissue (Figure 5-9).

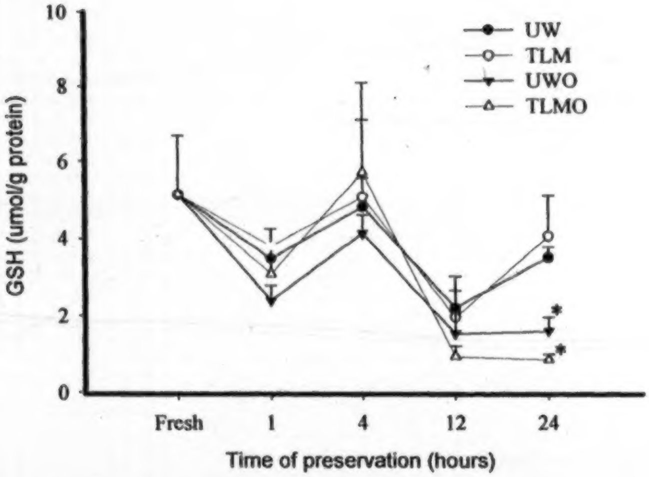


Figure 5-8: GSH level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, TLM: Two-Layer Method, UWO: UW with continuous bubbling of oxygen, TLMO: TLM with continuous bubbling of oxygen.* $p<0.001$ as compared to UW and TLM groups

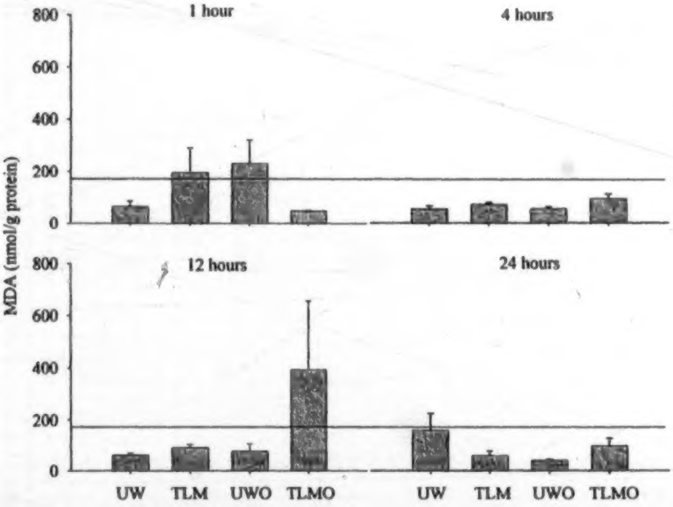


Figure 5-9: MDA level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, TLM: Two-Layer Method, UWO: UW with continuous bubbling of oxygen, TLMO: TLM with continuous bubbling of oxygen. Line represents the fresh value.

Apoptosis

DNA fragmentation level (ratio to fresh) of pancreatic tissue was significantly lower in UWO group (2.09±0.67) as compared to UW (6.27±0.12, p<0.001) group at 1 hour of pancreas preservation. It could decrease apoptosis by 66.5% in the first hour of preservation while TLM could just decrease it by 18.1%.. DNA fragmentation level of pancreatic tissue was significantly lower in TLM (5.13±0.44) as compared to UW (6.27±0.12, p<0.01) group at 1 hour of pancreas preservation but it was significantly higher as compared to UWO group and remained so during 12 hours of preservation. DNA fragmentation level of pancreatic tissue in UWO group was 45.8% less than that in TLM group (1.13±0.19 vs. 2.09±0.20, p<0.001) at 12 hours of pancreas preservation (Figure 5-10).

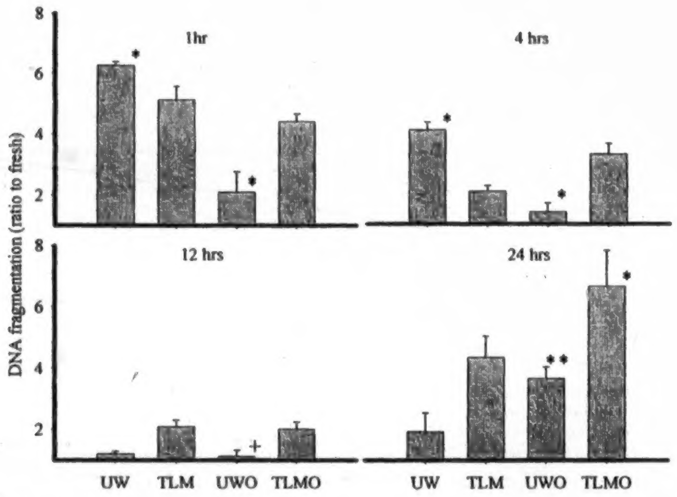


Figure 5-10: DNA fragmentation level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, TLM: Two-Layer Method, UWO: UW with continuous bubbling of oxygen, TLMO: TLM with continuous bubbling of oxygen. * p<0.01 as compared to other groups, + P<0.001 as compared to TLM and TLMO, ** p<0.01 as compared to UW.

Caspase 3 level (ratio to fresh) of pancreatic tissue was significantly lower in UWO group (0.63±0.18) as compared to UW (1.15±0.22, $p<0.01$) group at 1 hour of pancreas preservation (Figure 5-11). Continuous bubbling of oxygen could decrease caspase 3 by 45.2% in the first hour of preservation as well as TLM. Caspase 3 level of pancreatic tissue was also significantly lower in UWO (0.61±0.27) as compared to TLM (1.16±0.21, $p<0.01$) group at 12 hours of pancreas preservation which leads to 47.4% decrease in caspase 3 by UWO as compared to TLM.

Caspase 8 level (ratio to fresh) of pancreatic tissue was significantly higher in UWO group as compared to TLM group at 4 hours of pancreas preservation, however, it was significantly lower at 12 hours of preservation (Figure 5-12). Caspase 9 level (ratio to fresh) of pancreatic tissue was significantly lower in TLMO group as compared to TLM and UW groups at 1 and 12 hours of pancreas preservation. It was significantly higher in UWO group at 4 hours of preservation (Figure 5-13).

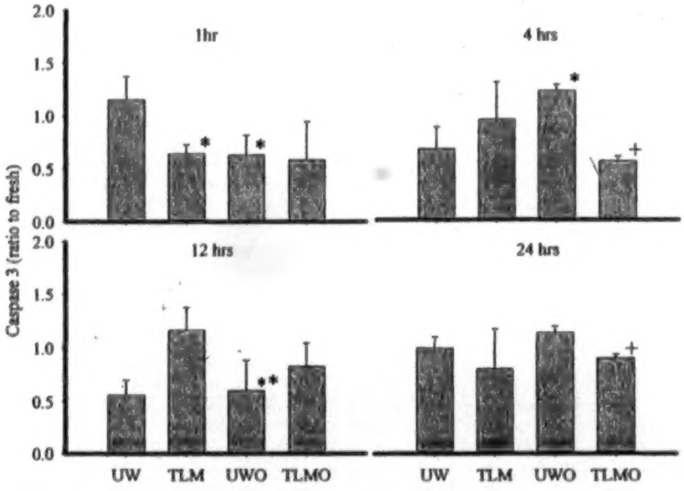


Figure 5-11: Caspase 3 level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, TLM: Two-Layer Method, UWO: UW with continuous bubbling of oxygen, TLMO: TLM with continuous bubbling of oxygen. * $p<0.01$ as compared to UW. ** $P<0.01$ as compared to TLM, + $P<0.01$ as compared to UWO.

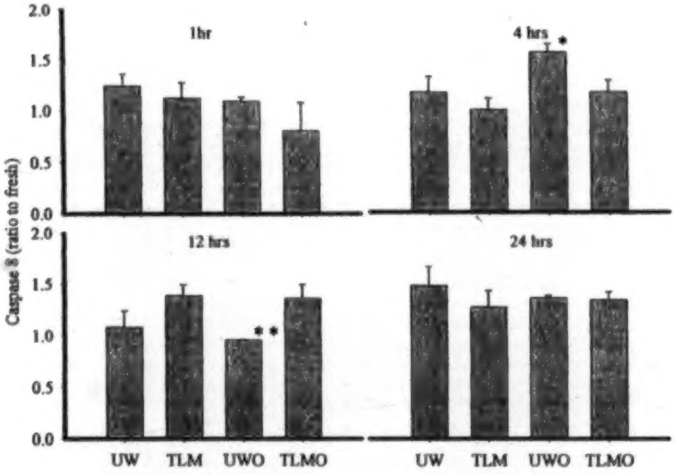


Figure 5-12: Caspase 8 level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, TLM: Two-Layer Method, UWO: UW with continuous bubbling of oxygen, TLMO: TLM with continuous bubbling of oxygen. * $p<0.001$ as compared to TLM, ** $p<0.001$ as compared to TLM and TLMO.

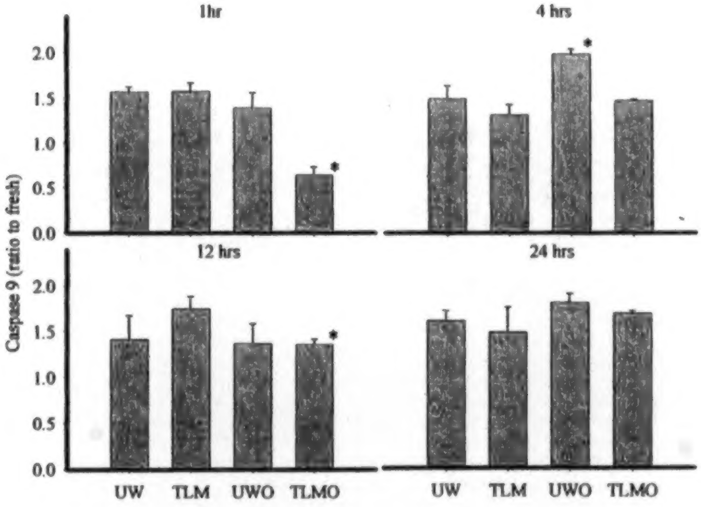


Figure 5-13: Caspase 9 level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, TLM: Two-Layer Method, UWO: UW with continuous bubbling of oxygen, TLMO: TLM with continuous bubbling of oxygen.* $p<0.001$ as compared to TLM.

Necrosis

LDH release level (ratio to fresh) of pancreatic tissue was significantly higher in UWO group (3.52±0.24) and TLMO group (3.97±0.19) as compared to UW (1.90±0.34, $p<0.001$) and TLM groups (1.77±0.09, $p<0.001$) at 1 hour of pancreas preservation, but not the afterwards. Continuous bubbling of oxygen in TLMO group did not show any significant difference in LDH release level as compared to continuous bubbling of oxygen in UWO group during 24 hours of pancreas preservation (Figure 5-14).

Figure 5-15A shows a normal fresh islet. The endocrine pancreas is separated from its exocrine counterpart by a capsule composed of reticular fibers. In hematoxylin- and eosin-stained sections, the endocrine cells are usually paler than the acinar cells. A number of hormones that function in carbohydrate metabolism are secreted by different types of sells, however it is not possible to resolve the different types of endocrine cells with this staining.

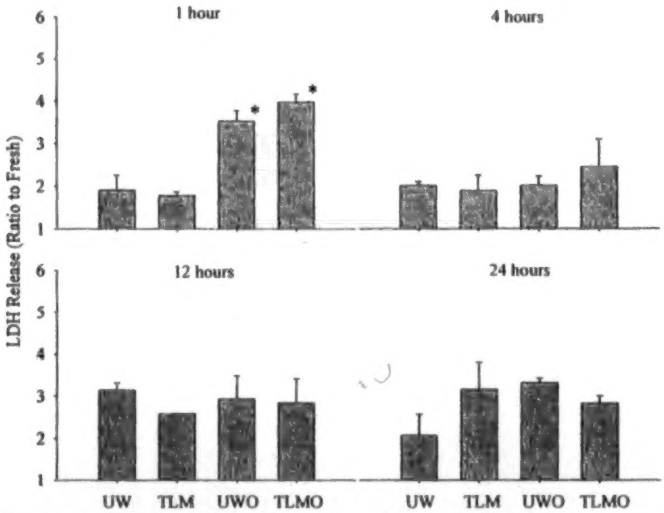


Figure 5-14: LDH release level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, TLM: Two-Layer Method, UWO: UW with continuous bubbling of oxygen, TLMO: TLM with continuous bubbling of oxygen. * $p<0.001$ as compared to UW and TLM groups

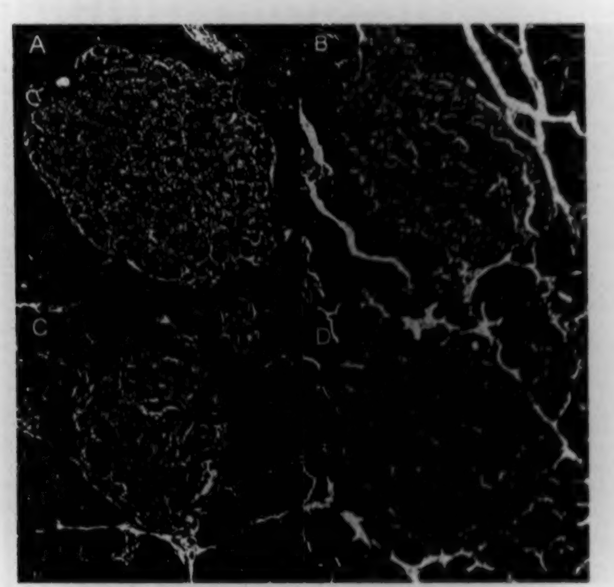


Figure 5-15: Hematoxylin and Eosin staining of pancreatic tissue containing an islet after 24 hours of preservation (magnification:*400); A: Fresh; B: Preserved in UW; C: in TLM; D: in UWO

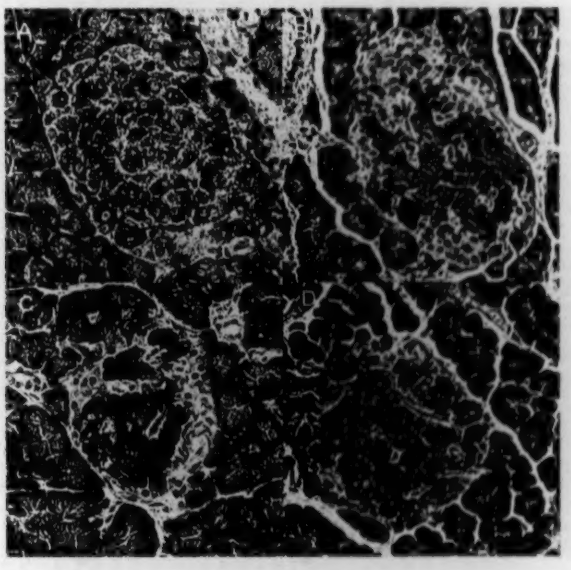


Figure 5-16: Insulin staining of pancreatic tissue containing an islet after 24 hours of preservation (magnification:*400); A: Fresh; B: Preserved in UW; C: in TLM; D: in UWO

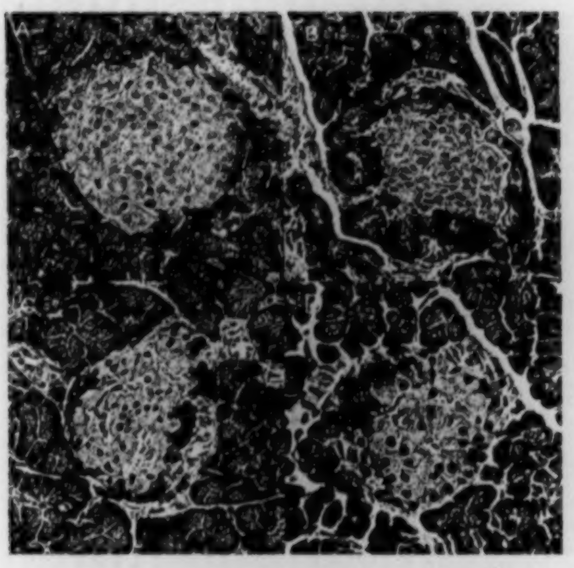


Figure 5-17: Glucagon staining of pancreatic tissue containing an islet after 24 hours of preservation (magnification:*400); A: Fresh; B: Preserved in UW; C: in TLM; D: in UWO

Preserving pancreas in UW solution for 24 hrs showed that almost all of central cells within the islet became heterochromatic, however peripheral cells looked normal (Figure 5-15 B). The darker-staining nuclei are those of cells that are not transcriptionally active. The structure of the capillaries has been totally destroyed and there was lots of shrinkage. Nucleoli were not seen and nucleus shape was changed. The basal areas of the pancreatic acinar cells contained stacks of rough endoplasmic reticulum which were still partially transcriptionally active. Heterochromatin started to be seen at 1 hour after preservation in UW solution; almost all of the β-cells were transcriptionally inactive at 12 hrs (Figures not shown) and remained unchanged until 24 hours after preservation.

In the two-layer method of preservation (Figure 5-15C), although the heterochromatin was seen less than that in UW solution preservation, most of the β-cells within the islet became heterochromatic. The structure of the capillaries has been destroyed and shrinkage was seen. Nucleoli were not seen and nucleus shape was changed. The endoplasmic reticulum of the pancreatic acinar cells were still partially transcriptionally active. Heterochromatin started to be seen at 4 hrs after preservation in TLM (Data not shown) and continued increasing until 24 hours after preservation.

UW oxygenation with continuous bubbling of oxygen showed that most of the cells within the islet remained euchromatic (Figure 5-15D). The structure of the capillaries remained intact and no shrinkage was seen intra-islet. Nucleoli were seen in some of the β-cells and nucleus shape was unchanged in many of the islets. The small percentage of heterochromatin started to be seen at 12 hours after preservation (data not shown) but remained unchanged until 24 hours after preservation. Immunocytochemical studies have shown that the two major cell types, α-cells and β-cells, are distributed in different locations within the islets. In general, β -cells tend to be located in the center of the islet (Figure 5-16) and α-cells around the periphery (Figure 5-17).

Islet Isolation Outcome

Post-purification islet yield was significantly higher in UWO group (1203±412 IEQ/pancreas) as compared to UW (116±94 IEQ/pancreas, $p<0.001$) and TLM groups (159±127 IEQ/pancreas, $p<0.001$) at 24 hours of pancreas preservation; but not to the fresh group (1262±379 IEQ/pancreas, Ns). Continuous bubbling of oxygen in TLMO group did not show any significant difference in post-purification islet yield as compared to continuous bubbling of oxygen in UWO group at 24 hours of pancreas preservation (Figure 5-18).

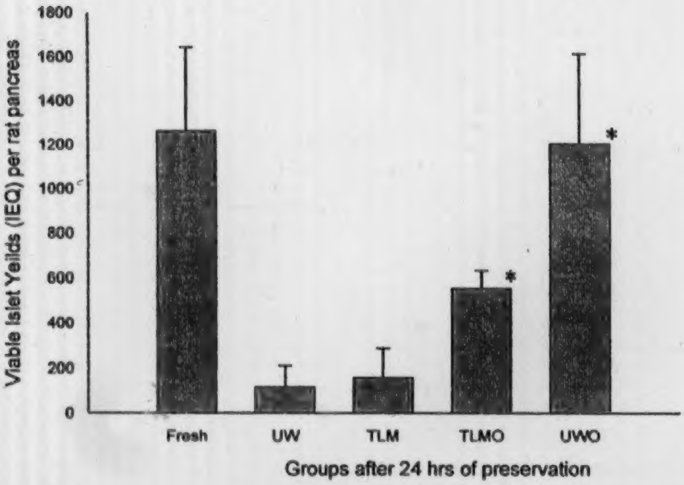


Figure 5-18: Post-purification viable islet yield after 24 hours of pancreas preservation in different groups. UW: University of Wisconsin solution, TLM: Two-Layer Method, UWO: UW with continuous bubbling of oxygen, TLMO: TLM with continuous bubbling of oxygen. * p<0.001 as compared to UW and TLM groups.

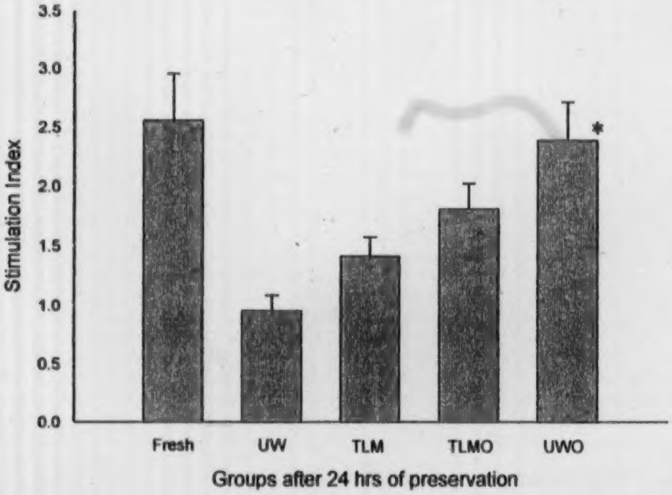


Figure 5-19: Stimulation index of isolated islets after 24 hours of pancreas preservation in different groups. UW: University of Wisconsin solution, TLM: Two-Layer Method, UWO: UW with continuous bubbling of oxygen, TLMO: TLM with continuous bubbling of oxygen. * p<0.001 as compared to UW and TLM groups

Stimulation index of isolated islets was significantly higher in UWO group (2.39±0.32) as compared to UW (0.95±0.13, $p<0.001$) and TLM groups (1.41±0.16, $p<0.001$) at 24 hours of pancreas preservation; but not to the fresh group (2.55±0.39, Ns). Continuous bubbling of oxygen in TLMO group did not show any significant difference in stimulation index of isolated islets as compared to continuous bubbling of oxygen in UWO group at 24 hours of pancreas preservation (Figure 5-19).

Discussion

In this study, dissolved oxygen level in UW solution with continuous bubbling of oxygen was higher as compared to pre-oxygenated TLM. In spite of the huge amount of dissolved oxygen in the PFC layer, it was released slowly and not completely to the UW solution in TLM group. Brandhost et al. determined partial oxygen tension in 500 mL of PFC stored for 30 hours at 4°C in a closed 1 L-wide mouth jar. PFC was pre-charged with 100% oxygen for 30 min at a rate of 2 L/min was stored alone (OLM, open circles, n=3) or covered with 300 mL of UW solution (TLM, filled squares,n=3) [4]. As shown in Figure 1-8, just a little amount of oxygen could be released from PFC to UW as compared to that from PFC to air. This could be explained by the high surface tension of UW as an aqueous solution (74.9 erg cm^{-2} at 5 °C).

In this study, the continuous bubbling of oxygen improved energy profile of pancreatic tissue during 24 hours of preservation comparable to the fresh values and switched the anaerobic metabolism to aerobic one effectively. The data shows that TLM also improves the pancreatic tissue energy profile after 12 hours of preservation however the values never reach to the fresh values and it cannot switch the anaerobic metabolism to aerobic one effectively. The two-layer method previously known to supply oxygen to the pancreas and allows ATP production during pancreas preservation and delays the process of cell death [15]. Ricordi et al. [16] demonstrated significantly improved islet recovery from marginal "older" donors by using TLM. Although the experimental numbers were not large, Matsumoto et al. [17] showed significantly improved islet recovery by using TLM after 6 to 8 hours of cold storage in UW solution with short and prolonged total cold storage time. Recently, Brandhost et al. presented compelling data that oxygenated perfluorocarbon can be used even in a one-layer method (OLM) without reducing the efficacy of the two layer method (TLM) preservation solution [4].

Lakey et al. previously reported some data concerning increased human islet recovery using two-layer method compared to simple UW cold storage [18, 19]. In spite of all mentioned benefits, there are still some concerns regarding the TLM. One of the potential drawback of PFCs (other than cost) is their relative immiscible nature and the high specific gravity (1.93 g/mL), which may result in physical injury attributed to forcing a delicate tissue under the surface of a dense immiscible liquid. The dependence of perfluorocarbons on Henry's Law of partial pressures allows the potential for increased oxygen availability. This fact of oxygen delivery also limits the effective use of perfluorocarbons to situations when the partial pressure of oxygen is supranormal and static [20]. In addition, oxygen in Perfluorocarbon (PFC) could not be delivered to the pancreas in a manner of direct diffusion through the undersurface of the pancreas during preservation, because the pancreas is not oxygenated when it is not in contact with oxygenated PFC [21].

Recently, investigators have demonstrated that ischemia and hypoxia are associated with an influx of Na^+ into red blood cells, cardiac myocytes, and capillary endothelial cells [22]. This influx of Na^+ is postulated to occur through activation of the Na^+-H^+ exchanger, which is stimulated by the switch from aerobic to anaerobic metabolism that occurs during hypoxia [23]. Activation of the Na^+-H^+ exchanger results in intracellular alkalinization [24]. Our data shows that TLM is not able to restore the activation of the Na^+-H^+ exchanger, while continuous bubbling of oxygen increases the pH of the solution. In a recently published paper, the PO_2 was measured using fiber optic sensors in the core of porcine pancreatic tissue preserved with the TLM in media saturated with 100% oxygen. Experimental measurements verified that PO_2 is virtually zero in the core of a 1-cm-thick pancreatic piece preserved with the TLM [25].

During hypothermic preservation pancreas is facing to two important deleterious effects: hypothermia and hypoxia. Hypothermia is used to reduce the energy demands and cellular oxygen consumption. A decrease in temperature from 37 °C to 4°C will decrease the metabolism by 12 fold which result in decrease of oxygen consumption by tissue [26]. In cases of severe O_2 limitation, most excitable cells of mammals cannot continue to meet the energy demands of active ion-transporting systems, leading to rapid exhaustion of fermentable substrate, catastrophic membrane failure and cell death [27]. On the other route, accumulation of lactic acid due to anaerobic glycolysis results in tissue acidosis which is deleterious to normal cell function. During ischemia, in addition to the usual pathway for lactic acid production, lactic dehydrogenase (LDH) metabolizes pyruvate to lactic acid. The high concentrations of lactic acid not only injure cells but also can activate macrophages, leading to cytokine production and the initiation of an inflammatory response [28]. On the other hand, cooling of tissues during hypothermic preservation has been shown to reduce the activity of the membrane ion exchange mechanisms, specifically the Na^+-K^+ ATPase system [29]. The loss of ionic regulation has been shown to lead to an intracellular gain of extra cellular sodium [30]. In any case, if cold-induced accumulation sodium continues increasing, the rise in cytosolic sodium will ultimately lead to membrane depolarization, the opening of voltage-dependant Ca^{2+} channels, rapid influx of calcium and initiation of membrane phospholipid hydrolysis [31].

Once initiated, the pathological series of effects leading to necrotic cell death during hypothermia is largely uncontrollable and analogous to the irreversible membrane injury. In spite of its deleterious effects, the current preferred method for long-term pancreas preservation is through reduced temperatures. Our data show that continuous bubbling of oxygen is not successful in preventing hypothermia induced necrosis.

Providing oxygen to a tissue leads to the production of free radicals that occurs via the hypoxanthine-xanthine oxidase reaction and can contribute to cell injury by participating in either protein peroxidation, direct DNA damage, or lipid peroxidation [32]. The data showed that there was no significant lipid peroxidation by tissue oxygenation, however, the GSH values decreased significantly with bubbling of oxygen that reveals existence of some levels of free radicals.

It has recently been reported that apoptosis occurs before transplantation through isolation processes, especially by the disruption of extracellular matrix surrounding islets [33] and intra-islet cytokine production mediated by exposure to endotoxin-contaminated reagents, [34] and that islet apoptosis can cause early graft loss after transplantation. [35,36] There are also other reports showing the improvement of islet graft function by protecting islets from apoptosis by transduction of anti-apoptotic genes, such as bcl-2 in islets. [37] Therefore, the protection of islets from apoptosis might be one of the clues to reduce graft loss in islet transplantation. It has been found that the apoptotic program is executed through 3 major pathways: the death-receptor, the mitochondrial, and mitogen-activated protein kinase (MAPK) pathways.[38] Isolated islets have been shown to lead to apoptosis through any of these 3 pathways. [39,40] In every case thus far reported, apoptosis in isolated islets occurs as a result of the isolation process, cytokine stimulation, or irradiation. There is one report shows that pancreas preservation before islet isolation has an influence on the occurrence of apoptosis in isolated islets. The authors demonstrated that isolated islets before transplantation sustained apoptosis at a high rate when the pancreas was preserved before islet isolation by conventional cold storage in UW solution compared with no preservation. However, apoptosis of isolated islets decreased significantly by using TLM as a preservation technique, although not to the degree as with no preservation.

Hypoxia has been shown to decrease the mitochondrial potential leading to the release of cytochrome C. The released cytochrome C binds with the Apaf1/caspase 9 complex to initiate apoptosis [41]. An observation indicate that preservation of pancreata in PFC reduces organ hypoxia during cold preservation, resulting in preservation of the mitochondrial redox potential and reduction in apoptosis mediated through the mitochondrial pathway [2]. Our data show that continuous bubbling of oxygen prevents apoptosis in a more effective and consistent way during 12 hours of preservations as compared to TLM. Apoptosis observed in pancreatic tissue was mainly mitochondria-dependent, partly mediated by change in caspase 9 initiated by hypoxia. Any extrinsic pathway (caspase 8) dependent change by hypoxia was not observed. Although the activation of caspase is most likely a predominant mechanism inducing apoptosis, there is accumulating evidence demonstrating that apoptosis could be mediated by mechanisms that do not involve caspases [42]. This pathway, termed caspase-independent apoptosis, is characterized by a large scale DNA fragmentation with an early chromatin condensation pattern [43]. This is in contrast to caspase-dependent apoptosis that is characterized by an oligonucleosomal DNA fragmentation with an advanced chromatin condensation pattern. It has previously been reported that various caspase-independent factors, such as apoptosis inducing factor (AIF), endonuclease G (Endo G) and High temperature requirement protein A2 (HtrA2/Omi), could induce apoptosis without mediation of caspases. These factors normally reside in the mitochondrial intermembrane space. However, in response to apoptotic stimuli, they are released from the mitochondria to the cytosol without concurrent caspase activation, translocate to the nucleus, and cause DNA fragmentation [44].

Although the effect of TLM on pancreas preservation has been documented to improve islet cell yield and function, few data are available concerning the benefit of TLM in human islet isolation. Lakey et al. previously reported some preliminary data concerning increased human islet recovery using TLM compared to simple UW cold storage and improved function from transplanted islets isolated through this technique [45]. However, they also found that transplanted mass and functional viability of islet isolated from TLM-preserved pancreas were similar to those from UW-preserved pancreas. Patients receiving the TLM-islet or the UW-islet showed a marked decrease in insulin requirement after transplantation. No significant difference was observed in a decrease in insulin requirement between patients receiving the TLM-islet and the UW-islet [46].In this study we found that continiuos bubbling of oxygen significantly improves islet yield and function after isolation as compared to TLM and comparable to fresh isolated islets.

Taken together, oxygenation of pancreas through bubbling shows superior results in islet yield, function, pancreatic tissue energy profile, and apoptosis blockade as compared to TLM or UW alone. However, bubbling of oxygen expose tissue to some degrees of free radicals and cannot prevent necrosis. Apoptosis improved in pancreatic tissue was mainly mitochondria-dependent, and partly mediated by change in caspase 9 initiated by hypoxia. Big changes of DNA fragmentation, small changes in caspases, and increase in mitochondrial function leads to thinking to another possible pathway, so called caspase-independent apoptosis (AIF, and EndoG) which needs to be further studied.

Bibliography

---

[1] Palombo JD, Pomposelli JJ, Hirschberg Y, Blackburn GL and Bistrian BR, Glycolytic support of adenine nucleotides in rat liver flush-preserved with UW or Collins' II. Importance of donor nutritional status. Transplantation 1989; 48: 901 905.

[2] Ramachandran S, Desai NM, Goers TA, Benshoff N, Olack B, Shenoy S, Jendrisak MD, Chapman WC, Mohanakumar T. Improved islet yields from pancreas preserved in perflurocarbon is via inhibition of apoptosis mediated by mitochondrial pathway. Am J Transplant. 2006 Jul;6(7):1696-703.

[3] Hiraoka K, Kuroda Y, Tanioka Y et al. The importance of adenosine and a colloid (Dextran 40) in resuscitation of ischemically damaged canine pancreas during preservation by the two-layer method. Transplantation 1994; 58: 1279-1281.

[4] Brandhorst D, Bretzel RG, Brandhorst H. Oxygen and pancreas preservation Transplantation. 2006 ;81(3):492

[5] Hering BJ, Matsumoto I, Sawada T, Nakano M, Sakai T, Kandaswamy R, Sutherland DE. Impact of two-layer pancreas preservation on islet isolation and transplantation. Transplantation. 2002 Dec 27;74(12):1813-6

[6] Matsumoto S, Kuroda Y, Hamano M, Kim Y, Suzuki Y, Ku Y, Saitoh Y. Direct evidence of pancreatic tissue oxygenation during preservation by the two-layer method. Transplantation. 1996 Dec 15;62(11):1667-70.

[7] Matsumoto S, Rigley TH, Qualley SA, Kuroda Y, Reems JA, Stevens RB. Efficacy of the oxygen-charged static two-layer method for short-term pancreas preservation and islet isolation from nonhuman primate and human pancreata. Cell Transplant. 2002;11(8):769-77.

[8] Kuroda Y, Sakai T, Suzuki Y, Tanioka Y, Matsumoto S, Kim Y, Fujita H, Hamano M, Hasegawa Y, Ku Y, Saitoh Y. Small bowel preservation using a cavitary two-layer (University of Wisconsin solution/perfluorochemical) cold storage method. Transplantation. 1996 Feb 15;61(3):370-3.

[9] Passonneau JV, Lowry OH. Enzymatic Analysis. A Practical Guide. Totowa, NJ: Humana Press, 1993.

[10] Fernandez-Checa JC, Kaplowitz N. The use of monochlorobimane to determine hepatic GSH levels and synthesis. Anal Biochem 1990 Nov 1;190(2):212-9.

[11] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351-358.

[12] Holdenrieder S, Stieber P, Bodenmuller H, Busch M, et al. Quantification of nucleosomes in serum by the cell death detection ELISA plus, Biochemica 2002; 1: 25-27

[13] Nicholson, D.W., et al., Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature, 376, 37-43 (1995).

[14] Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J, Protein measurement with the Folin phenol reagent, J Biol Chem 1951; 193: 265-75.

[15] Fujino Y, Kuroda Y, Morita A, Tanioka Y, Ku Y, Saitoh Y. The effect of fasting and exogenous adenosine on ATP tissue concentration and viability of canine pancreas

grafts during preservation by the two-layer method. Transplantation 1993; 56: 1083-1086.

[16] Ricordi C, Fraker C, Szust J, Al-Abdullah I, Poggioli R, Kirlew T, Khan A, Alejandro R. Improved human islet isolation outcome from marginal donors following addition of oxygenated perfluorocarbon to the cold-storage solution. Transplantation. 2003; 75(9):1524-7.

[17] Matsumoto S, Qualley SA, Goel S, Hagman DK, Sweet IR, Poitout V, Strong DM, Robertson RP, Reems JA. Effect of the two-layer (University of Wisconsin solution-perfluorochemical plus O2) method of pancreas preservation on human islet isolation, as assessed by the Edmonton Isolation Protocol. Transplantation. 2002; 74(10):1414-9.

[18] Lakey JR, Tsujimura T, Shapiro AM, Kuroda Y. Preservation of the human pancreas before islet isolation using a two-layer (UW solution-perfluorochemical) cold storage method. Transplantation 2002; 74: 1809-11.

[19] Tsujimura T, Kuroda Y, Avila JG, Kin T, Oberholzer J, Shapiro AM, Lakey JR. Influence of pancreas preservation on human islet isolation outcomes: impact of the two-layer method. Transplantation 2004; 78: 96-100.

[20] Tsujimura T, Salehi P, Walker J, Avila J, Madsen K, Lakey J, Kuroda Y, Churchill TA. Ameliorating small bowel injury using a cavitary two-layer preservation method with perfluorocarbon and a nutrient-rich solution. Am J Transplant. 2004 Sep;4(9):1421-8.

[21] Kuroda Y, Fujino Y, Kawamura T, et al. Mechanism of oxygenation of canine pancreas during preservation by a two-layer(Euro-Collins solution/perfluorochemical) cold-storage method. Transplantation 1990; 49: 694.

[22] Haigney MCP, Miyata H, Lakatta EG, Stern MD, Silverman HS. Dependence of hypoxic cellular calcium loading on Na+-Ca2+ exchange. *Circ Res.* 1992;71:547-557.

[23] Mazzoni MC, Intaglietta M, Cragoe EJ, Arfors KE. Amiloride-sensitive Na+ pathways in capillary endothelial cell swelling during hemorrhagic shock. *J Appl Physiol.* 1992;73:1467-1473.

[24] Frelin C, Vigne P, Ladoux A, Lazdunski M. The regulation of the intracellular pH in cells from vertebrates. *Eur J Biochem.* 1988;174:3-14

[25] Papas KK, Hering BJ, Gunther L, Rappel MJ, Colton CK, Avgoustiniatos ES. Pancreas oxygenation is limited during preservation with the two-layer method. Transplant Proc. 2005; 37(8):3501-4.

[26] Belzer FO, Southard JH. Principles of solid-organ preservation by cold storage. Transplantation. 1988; 45(4): 673-6.

[27] Boutilier RG. Mechanisms of cell survival in hypoxia and hypothermia. J Exp Biol. 2001 Sep; 204(Pt 18):3171-81

[28] Kellum JA, Song M, Li J. Lactic and hydrochloric acids induce different patterns of inflammatory response in LPS-stimulated RAW 264.7 cells. Am J Physiol Regul Integr Comp Physiol. 2004; 286(4):R686-92.

[29] Martin DR, Scott DF, Downes GL, Belzer FO. Primary cause of unsuccessful liver and heart preservation: cold sensitivity of the ATPase system. Ann Surg. 1972; 175(1): 111-7.

[30] Wang LCH. Ecological, physiological and biological aspects of torpor in mammals and birds. In: Wang L. ed. Advances in comparative and environmental physiology. Berlin, Springer, 1989: 361

[31] Hochachka PW. Defense strategies against hypoxia and hypothermia. Science. 1986; 231(4735):234-41.

[32] Boggi U, Vistoli F, Del Chiaro M, Signori S, Croce C, Pietrabissa A, Berchiolli R, Marchetti P, Prato SD, Mosca F. Pancreas preservation with University of Wisconsin and Celsior solutions: a single-center, prospective, randomized pilot study. Transplantation. 2004; 77(8):1186-90.

[33] F. Thomas, J. Wu, J.L. Contreras, C. Smyth, G. Bilbao, J. He and J. Thomas, A tripartite anoikis-like mechanism causes early isolated islet apoptosis. Surgery 130 (2001), pp. 333 338

[34] T. Bemey, R.D. Molano, P. Cattan, A. Pileggi, C. Vizzardelli, R. Oliver et al., Endotoxin-mediated delayed islet graft function is associated with increased intra-islet cytokine production and islet cell apoptosis. Transplantation 71 (2001), pp. 125 132.

[35] S. Paraskevas, D. Maysinger, R. Wang, T.P. Duguid and L. Rosenberg, Cell loss in isolated human islets occurs by apoptosis. Pancreas 20 (2000), pp. 270–276.

[36] A.M. Davalli, L. Scaglia, D.H. Zangen, J. Hollister, S. Bonner-Weir and G.C. Weir, Vulnerability of islets in the immediate posttransplantation period. Dynamic changes in structure and function. Diabetes 45 (1996), pp. 1161 1167

[37] I.L. Contreras, G. Bilbao, C.A. Smyth, X.L. Jiang, D.E. Eckhoff, S.M. Jenkins et al., Cytoprotection of pancreatic islets before and soon after transplantation by gene transfer of the anti-apoptotic Bcl-2 gene. Transplantation 71 (2001), pp. 1015 1023.

[38] M.O. Hengartner, The biochemistry of apoptosis. Nature 407 (2000), pp. 770 776.

[39] S. Paraskevas, R. Aikin, D. Maysinger, J.R. Lakey, T.J. Cavanagh, B. Hering et al., Activation and expression of ERK, JNK, and p38 MAP-kinases in isolated islets of Langerhans: implications for cultured islet sundval. FEBS Lett 455 (1999), pp. 203 208

[40] S. Paraskevas, R. Aikin, D. Maysinger, J.R. Lakey, T.J. Cavanagh, D. Agapitos et al., Modulation of JNK and p38 stress activated protein kinases in isolated islets of Langerhans: insulin as an autocrine survival signal. Ann Surg 233 (2001), pp. 124 133

[41] Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997; 91: 479 489.

[42] Abraham MC, Shaham S. Death without caspases, caspases without death.

Trends Cell Biol. 2004 Apr;14(4):184-93.

[43] Penninger JM, Kroemer G. Mitochondria, AIF and caspases--rivaling for cell death execution. Nat Cell Biol. 2003 Feb;5(2):97-9.

[44] Lockshin RA, Zakeri Z. Caspase-independent cell death? Oncogene. 2004 Apr 12;23(16):2766-73.

[45] Lakey JR, Tsujimura T, Shapiro AM, Kuroda Y. Preservation of the human pancreas before islet isolation using a two-layer (UW solution-perfluorochemical) cold storage method. Transplantation 2002; 74: 1809-11.

---

[46] Kin T, Mirbolooki M, Salehi P, Tsukada M, O'Gorman D, Imes S, Ryan EA, Shapiro AM, Lakey JRT. Islet isolation and transplantation outcomes of pancreas preserved with University of Wisconsin solution versus two-layer method using pre-oxygenated perfluorocarbon. Transplantation . 2006: 82(10):

Chapter Six

Glucose Supplement in UW Solution

Effects and Mechanisms

Introduction

It has been observed that β cells are more sensitive to various apoptotic stimuli, such as the stress of low glucose [1,2] or hypoxia [3]. As an important cellular organelle, mitochondria exist in most eukaryotic cells in varying numbers [4]. A great deal of evidence has implicated the mitochondria as important regulators of apoptosis. Thus, in addition to their role as cellular components equipped with various oxidative and biosynthetic pathways and serving as a power plant for cellular ATP supply, they are the site of integration of cellular metabolism and apoptosis [5]. Although the importance of mitochondria in β cells for insulin secretion has long been recognized [6,7] their link with apoptotic proneness of β cells is less clear.

Stimulation of pancreatic β-cells with glucose induces an initial rise in β-cell energy metabolism, which leads to the closure of ATP-sensitive K^+ channels, plasma membrane depolarization, Ca^{2+} influx, and a rise in the cytosolic Ca^{2+} concentration that triggers insulin secretion [8]. Besides these acute effects on β-cell function, glucose also chronically exerts pleiotropic effects in pancreatic β-cells. Previous studies have indicated that the viability of cultured β-cells in 37°C depends on the prevailing glucose concentration, with an optimal glucose concentration for rodent β-cell survival of 10 mM [9,10]. Culture of β-cells at suboptimal glucose concentrations (<10 mM) triggers their apoptosis, suggesting that glucose suppresses an apoptosis program in these cells [1]. However, the mechanism leading to apoptosis induction under glucose limitation remains undefined. In this study the effects of glucose supplement in UW solution during hypothermic pancreas preservation were evaluated on islet survival and the probable mechanisms involved.

Methods

Pancreata were procured from male Sprague-Dawley rats according to standardized procurement procedures and after 10 ml intraductal injection of filtered UW, and preserved for 24 hours in four different groups including: UW solution, UW supplemented with 5mM glucose (UWG), UW with continuous bubbling of oxygen (UWO), and UW supplemented with 5mM glucose and continuous bubbling of oxygen (UWGO). Pre-oxygenation was done for 10 minutes with 100% oxygen at a flow rate of 2 L/min and continuous 100% oxygen bubbling in a flow rate of 0.2 L/min during 24 hours of preservation. Tissue samples (100 mg) were collected at 0, 1, 4, 12, and 24 hours after procurement. To arrest metabolic activity, samples were snap-frozen in liquid nitrogen and subsequently stored at -80 °C until processed. Aliquots of neutralized extracts were processed via standard enzyme-linked metabolite assays [11]. Spectrophotometric analysis was then performed to measure the absorbance of NADH at 340 nm, providing quantification of ATP, ADP, AMP, and lactate. Protein was measured according to Lowry et al [12]. Reduced Glutathione (GSH) was quantitatively measured by using monochlorobimane [13] and the amounts of Malondialdehyde (MDA) as according to Ohkawa [14]. DNA fragmentation was measured based on the quantitative "sandwich enzyme immunoassay" principle using mouse monoclonal antibodies directed against DNA and histones. The caspase 3, 8, and 9 colorimetric assays were based on the hydrolysis of the peptide substrate by caspases, resulting in the release of the p-nitroaniline (pNA) moiety [15]. Biopsies were stained for structure (Hematoxylin & Eosin).

Solution's pH was measured by symphony SB21 pH meter (VWR Scientific products). The Machine was calibrated to measure pH at 4°C. Necrosis was quantified based on the measurement of activity of lactate dehydrogenase (LDH) released from damaged cells. The partial oxygen pressure in UW was measured using a Foxy fiber optic oxygen sensor (Ocean Optics, Inc., FL).

Rat pancreata were digested with collagenase and purified by Ficoll-density gradients centrifugation. Dithizone-stained samples were evaluated post-purification in duplicate to determine islet yield. A sample of islet preparations was stained using SYTO Green (SYTO-13; Molecular Probes, Eugene, OR) and ethidium bromide. For assessment of *in vitro* function, islet preparations (25 islets) cultured at 37°C were examined for their secretory responsiveness to glucose during a static incubation. Stimulation index (SI) was calculated by dividing insulin output during high-glucose (20 mM) incubation divided by insulin output during basal-glucose (2.8 mM) incubation.

Metabolite data were presented as means ± SEM. Statistical differences between groups were determined using ANOVA followed by a post-hoc test (Tukey's).

Results

Oxygen Partial Pressure in UW solution

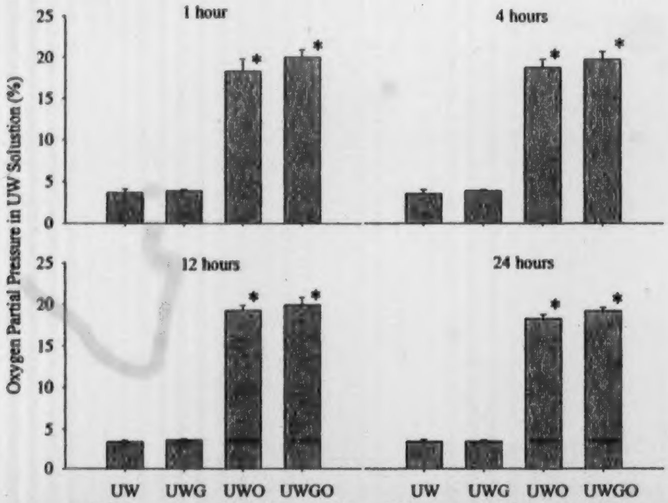


Figure 6-1: Oxygen partial pressure in UW solution (%) during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, UWG: UW supplemented with 5mM glucose, UWO: UW with continuous bubbling of oxygen, UWGO: UW supplemented with 5mM glucose and continuous bubbling of oxygen. * $p<0.001$ as compared to UW and UWG groups

Oxygen partial pressure in UW solution was significantly higher in UWGO (20.07±0.86%) and UWO group (19.35±0.84%) as compared to UW (3.58±0.35%, $p<0.001$) and UWG group (3.87±0.18%, $p<0.001$) at 1 hour of preservation and remained so afterwards (Figure 6-1).

Intracellular Energy Profile

ATP level of pancreatic tissue was significantly higher in UWGO (12.97±1.92 umol/g protein) and UWO group (15.67±3.40 umol/g protein) as compared to UW (4.65±0.42 umol/g protein, $p<0.001$) and UWG groups (6.07±2.38 umol/g protein, $p<0.001$) at 4 hours of pancreas preservation and afterwards. Glucose (5mM) in UWGO group showed no significant improvement in ATP level as compared to UWO group during 24 hours of pancreas preservation (Figure 6-2).

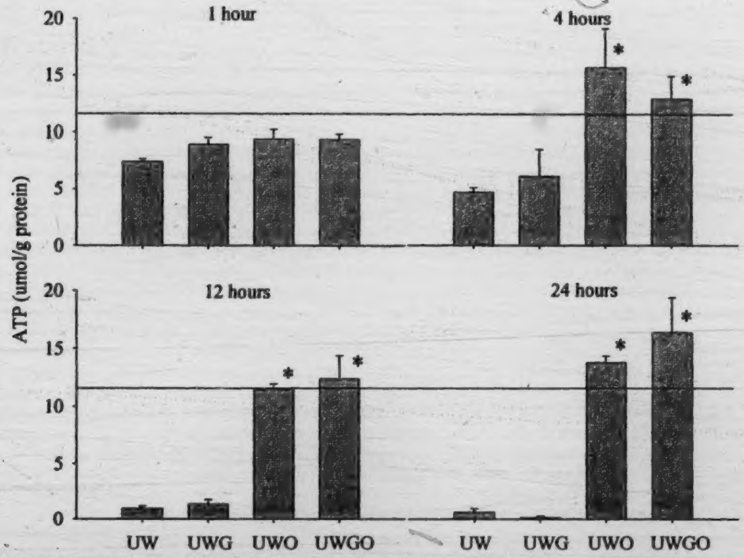


Figure 6-2: ATP level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, UWG: UW supplemented with 5mM glucose, UWO: UW with continuous bubbling of oxygen, UWGO: UW supplemented with 5mM glucose and continuous bubbling of oxygen. Line represents the fresh value.* $p<0.001$ as compared to UW and UWG groups

There was no significant difference in total adenylates levels between the groups and as compared to fresh procured tissue during the whole preservation time (Figure 6-3). Glucose (5mM) in UWGO group showed no significant improvement in total adenylates level as compared to UWO group during 24 hours of pancreas preservation. Energy charge (EC) level of pancreatic tissue was significantly higher in UWGO group (0.95±0.04) as compared to UW (0.64±0.02, $p<0.001$) and UWG (0.75±0.02, $p<0.001$) at 1 hour of pancreas preservation and afterwards and UWO groups (0.81±0.06, $p<0.05$) at 1 hour of pancreas preservation (Figure 6-4). Glucose (5mM) in UWGO group showed no significant improvement in energy charge level as compared to UWO group at 4 hours of pancreas preservation and afterwards. In both UWO and UWGO groups, there was no significant difference in EC levels as compared to fresh procured tissue at 24 hours of pancreas preservation.

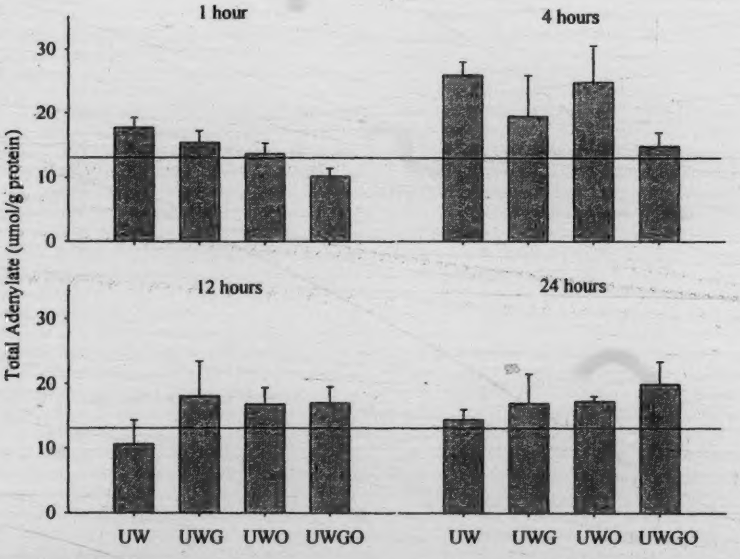


Figure 6-3: Total adenylates level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, UWG: UW supplemented with 5mM glucose, UWO: UW with continuous bubbling of oxygen, UWGO: UW supplemented with 5mM glucose and continuous bubbling of oxygen. Line represents the fresh value.

ADP/ATP ratio level of pancreatic tissue was significantly lower in UWGO (0.04±0.06) as compared to UW (1.05±0.10, p<0.001) and UWG groups (0.58±0.08, p<0.001) at 1 hour of pancreas preservation and afterwards. Glucose in UWGO group showed no significant difference in ADP/ATP ratio level as compared to UWO group during 24 hours of pancreas preservation (Figure 6-5).

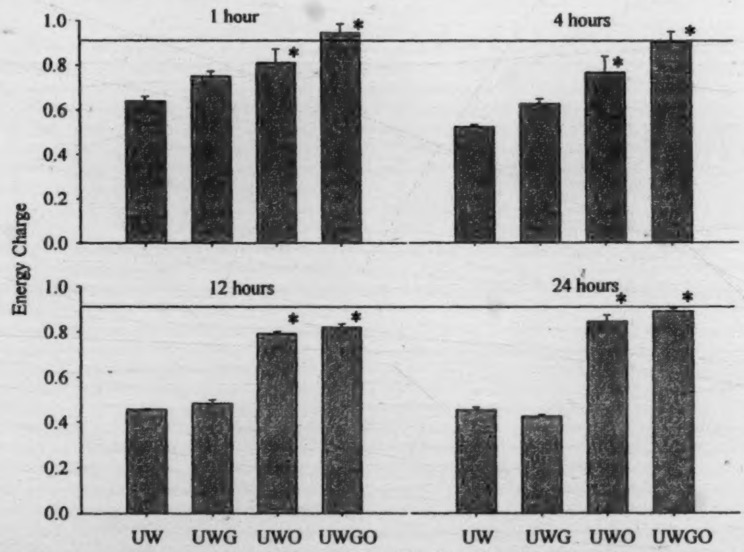


Figure 6-4: Energy charge level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, UWG: UW supplemented with 5mM glucose, UWO: UW with continuous bubbling of oxygen, UWGO: UW supplemented with 5mM glucose and continuous bubbling of oxygen.Line represents the fresh value. * p<0.001 as compared to UW and UWG groups

In both UWO and UWGO groups, there was no significant difference in ADP/ATP ratio levels as compared to fresh procured tissue at 24 hours of pancreas preservation.

Anaerobic Metabolism

Lactate level of pancreatic tissue was significantly lower in UWGO group (3.44±0.49 umol/g protein) as compared to UW (5.79±0.61 umol/g protein, p<0.001) and UWG groups (4.9±1.80, umol/g protein p<0.001) at 1 hour of pancreas preservation and afterwards (Figure 6-6). Glucose in UWG group showed no significant increase in lactate level as compared to UW group. It did not show any significant increase in UWGO group as compared to UWO group at 1 and 4 hours of preservation, however, it increased significantly afterwards.

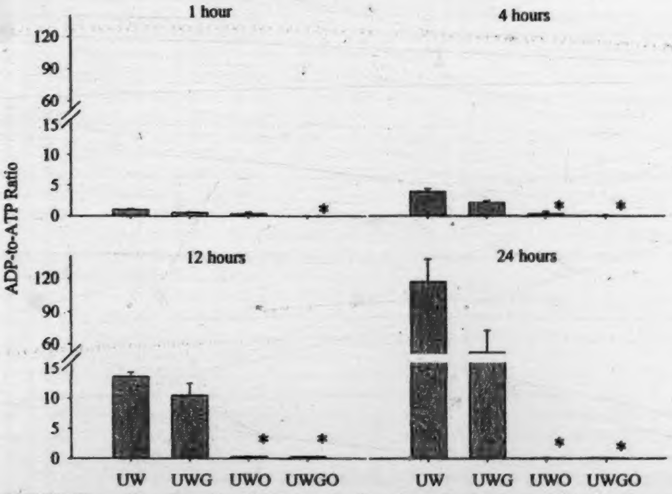


Figure 6-5: ADP/ATP ratio level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, UWG: UW supplemented with 5mM glucose, UWO: UW with continuous bubbling of oxygen, UWGO: UW supplemented with 5mM glucose and continuous bubbling of oxygen. * p<0.001 as compared to UW and UWG groups

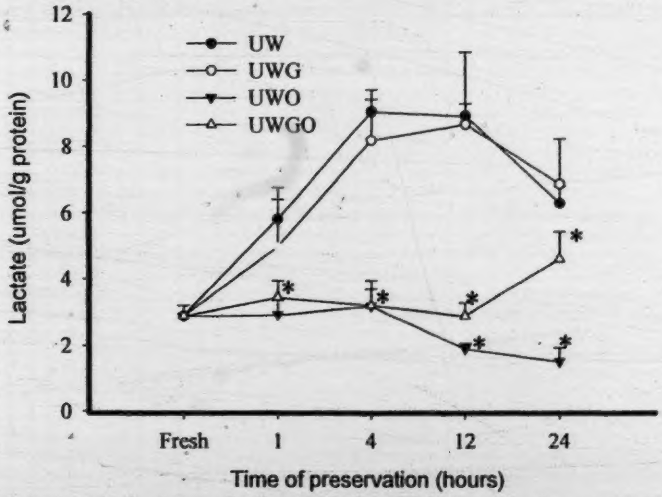


Figure 6-6: Lactate level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, UWG: UW supplemented with 5mM glucose, UWO: UW with continuous bubbling of oxygen, UWGO: UW supplemented with 5mM glucose and continuous bubbling of oxygen. * p<0.001 as compared to UW and UWG groups.

In UWO group, there was a significant decrease in lactate levels as compared to fresh procured tissue at 12 and 24 hours of pancreas preservation.

There was a significant decrease in UW solution's pH levels in UWG group as compared to those of fresh solution and other groups. Starting at 4 hours of preservation, there was a significant increase in UW solution's pH levels in UWGO group as compared to those of fresh solution, UWG and UW groups (Figure 6-7). However, it was significantly lower than that of UWO group at 12 and 24 hours.

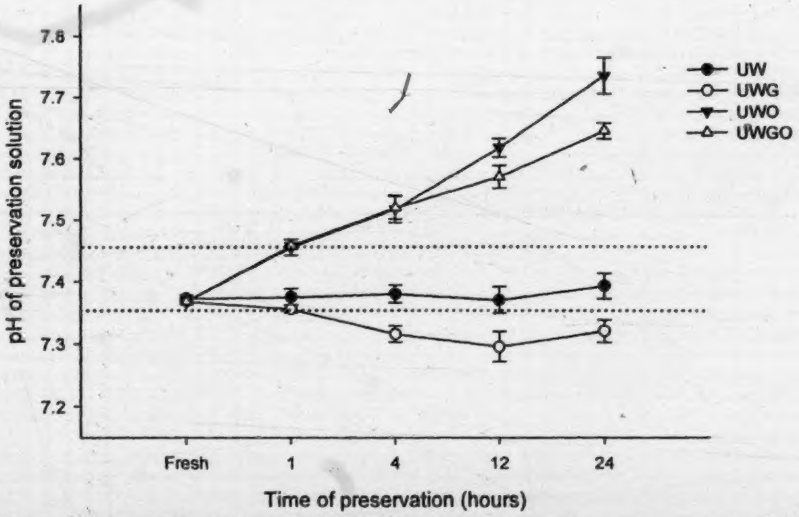


Figure 6-7: pH level of UW solution during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, UWG: UW supplemented with 5mM glucose, UWO: UW with continuous bubbling of oxygen, UWGO: UW supplemented with 5mM glucose and continuous bubbling of oxygen. Dot lines represent Upper and Lower limits of pH in a normal condition.

Oxidative Stress

GSH level of pancreatic tissue was significantly higher in UWGO (7.19±0.62 umol/g protein) and UWG groups (5.97±0.49 umol/g protein) as compared to UWO group (2.39±0.40 umol/g protein) and UW (3.48±0.76 umol/g protein, $p<0.001$) at 1 hour of pancreas preservation and afterwards. UWG and UWGO groups showed no significant difference in GSH level as compared to fresh pancreatic tissue during 24 hours of pancreas preservation (Figure 6-8).

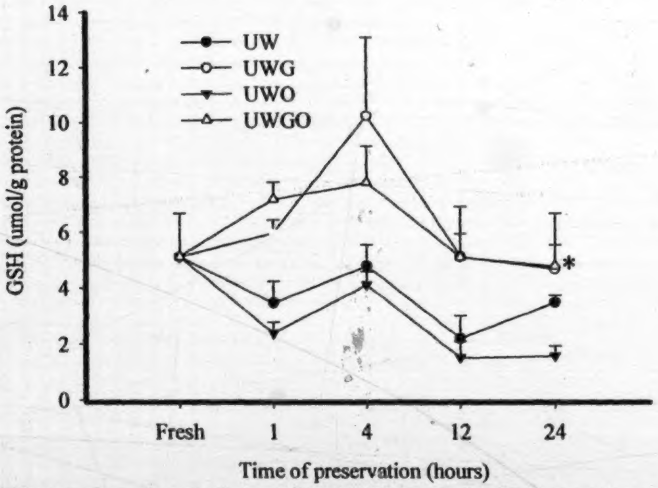


Figure 6-8: GSH level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, UWG: UW supplemented with 5mM glucose, UWO: UW with continuous bubbling of oxygen, UWGO: UW supplemented with 5mM glucose and continuous bubbling of oxygen. * $p<0.001$ as compared to UW and UWO group

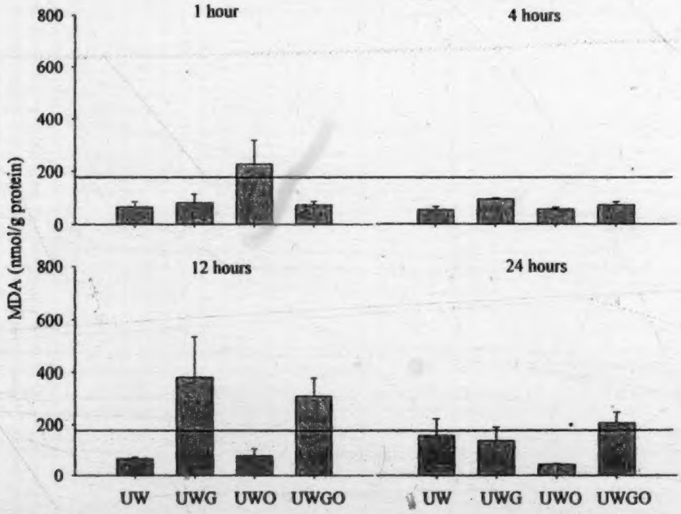


Figure 6-9: MDA level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, UWG: UW supplemented with 5mM glucose, UWO: UW with continuous bubbling of oxygen, UWGO: UW supplemented with 5mM glucose and continuous bubbling of oxygen. Line represents the fresh value.

There was also no significant difference in MDA levels between all groups during 24 hours of pancreas preservation and no significant increase as compared to fresh pancreatic tissue (Figure 6-9).

Apoptosis

DNA fragmentation level (ratio to fresh) of pancreatic tissue was significantly lower in UWGO (0.86±0.07) and UWG groups (0.34±0.05) as compared to UW (6.27±0.12, p<0.001) and UWO (2.09±0.67, p<0.001) groups at 1 hour of pancreas preservation. Glucose (5mM) in UWGO group showed a significant increase in DNA fragmentation level as compared to other groups at 24 hours of pancreas preservation (Figure 6-10). Glucose supplement to continuous bubbling of oxygen could decrease apoptosis by a 58.85% extra with a decrease of 86.2% in total in the first hour of preservation. DNA fragmentation level of pancreatic tissue in UWGO group remained 73.7% less than that in UWO group at 24 hours of pancreas preservation.

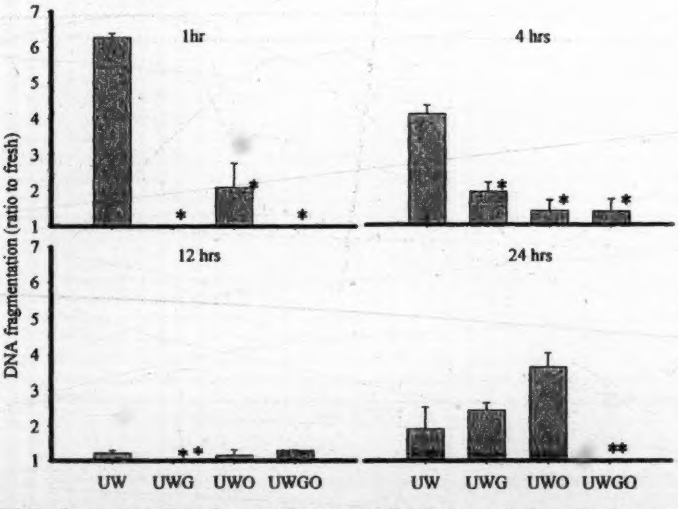


Figure 6-10: DNA fragmentation level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, UWG: UW supplemented with 5mM glucose, UWO: UW with continuous bubbling of oxygen, UWGO: UW supplemented with 5mM glucose and continuous bubbling of oxygen. * p<0.001 as compared to UW at 1 and 4 hours ** p<0.001 as compared to other groups at 12 and 24 hours

Caspase 3 level (ratio to fresh) of pancreatic tissue was significantly lower in UWO (0.63±0.18) and UWGO groups (0.63±0.18) as compared to UW (1.15±0.22, p<0.01) and UWG (1.15±0.22, p<0.01) groups at 1 hour of pancreas preservation (Figure 6-11). It was significantly lower in UWG (0.63±0.18) and UWGO groups (0.63±0.18) as compared to UWO groups at 4 hours of pancreas preservation. It remained significantly lower in UWGO group (0.63±0.18) as compared to other groups at 24 hours of pancreas preservation which leads to 36.4% less caspase 3 than that in UWO group at 24 hours of pancreas preservation. Caspase 8 level (ratio to fresh) of pancreatic tissue was significantly lower in UWGO and UWG groups as compared to UW and UWO groups at 4 and 24 hours of pancreas preservation (Figure 6-12).

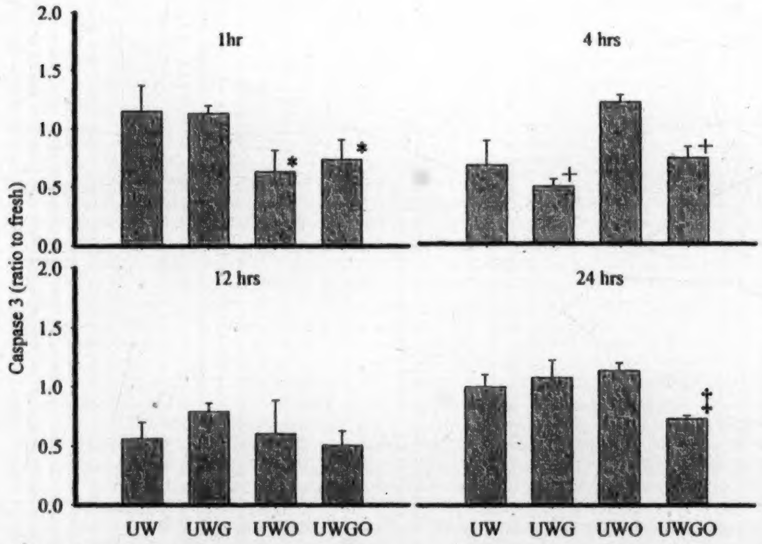


Figure 6-11: Caspase 3 level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, UWG: UW supplemented with 5mM glucose, UWO: UW with continuous bubbling of oxygen, UWGO: UW supplemented with 5mM glucose and continuous bubbling of oxygen. * $p<0.001$ as compared to UW and UWG. + $p<0.001$ as compared to UW and UWO. ‡ $p<0.001$ as compared to other groups

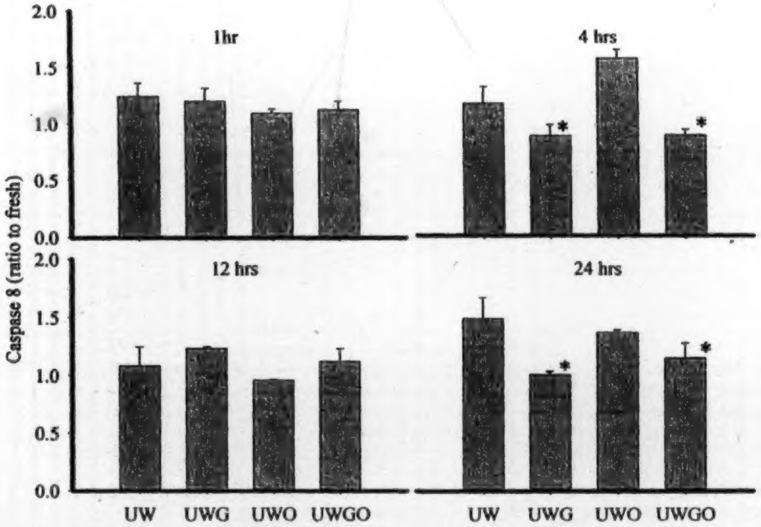


Figure 6-12: Caspase 8 level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, UWG: UW supplemented with 5mM glucose, UWO: UW with continuous bubbling of oxygen, UWGO: UW supplemented with 5mM glucose and continuous bubbling of oxygen. * p<0.001 as compared to UW and UWO groups

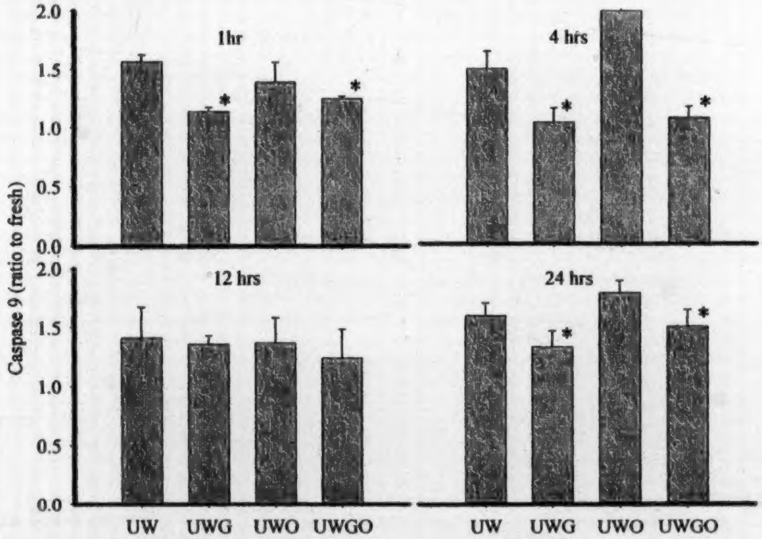


Figure 6-13: Caspase 9 level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, UWG: UW supplemented with 5mM glucose, UWO: UW with continuous bubbling of oxygen, UWGO: UW supplemented with 5mM glucose and continuous bubbling of oxygen. * p<0.001 as compared to UW and UWO groups

Caspase 9 level (ratio to fresh) of pancreatic tissue was significantly lower in UWGO and UWG groups as compared to UW and UWO groups at 1, 4 and 24 hours of pancreas preservation (Figure 6-13).

Necrosis

Glucose (5mM) in UWGO group showed a significant decrease in LDH release level as compared to UWO group at 1 hour of pancreas preservation. However, LDH release level (ratio to fresh) of pancreatic tissue was significantly higher in UWGO (4.99±0.42) and UWG groups (5.27±0.22) as compared to UW (3.14±0.22, p<0.001) and UWO groups (2.92±0.78, p<0.001) at 12 hours of pancreas preservation (Figure 6-14).

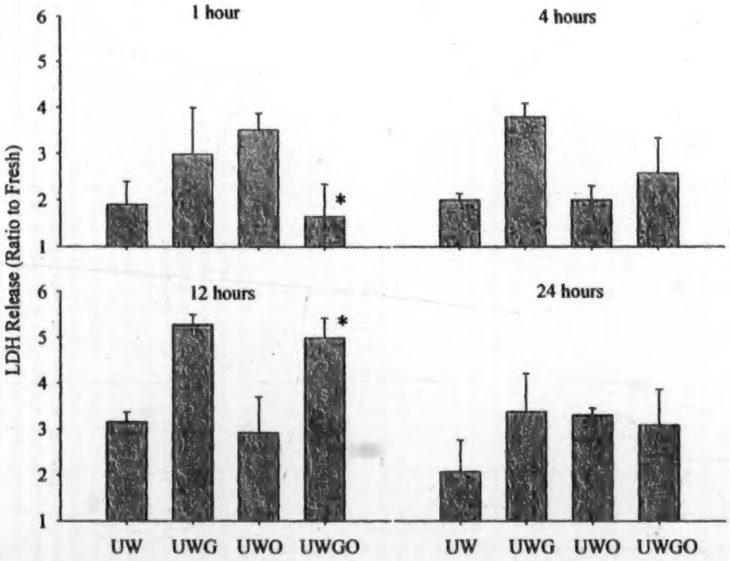


Figure 6-14: LDH release level of pancreatic tissue during 24 hours of rat pancreas preservation. UW: University of Wisconsin solution, UWG: UW supplemented with 5mM glucose, UWO: UW with continuous bubbling of oxygen, UWGO: UW supplemented with 5mM glucose and continuous bubbling of oxygen. * p<0.001 as compared to UWO group at 1 hour. * p<0.001 as compared to UW and UWO groups at 12 hours

The endocrine pancreas is separated from its exocrine counterpart by a capsule composed of reticular fibers. Figure 6-15F shows a normal fresh islet. The endocrine

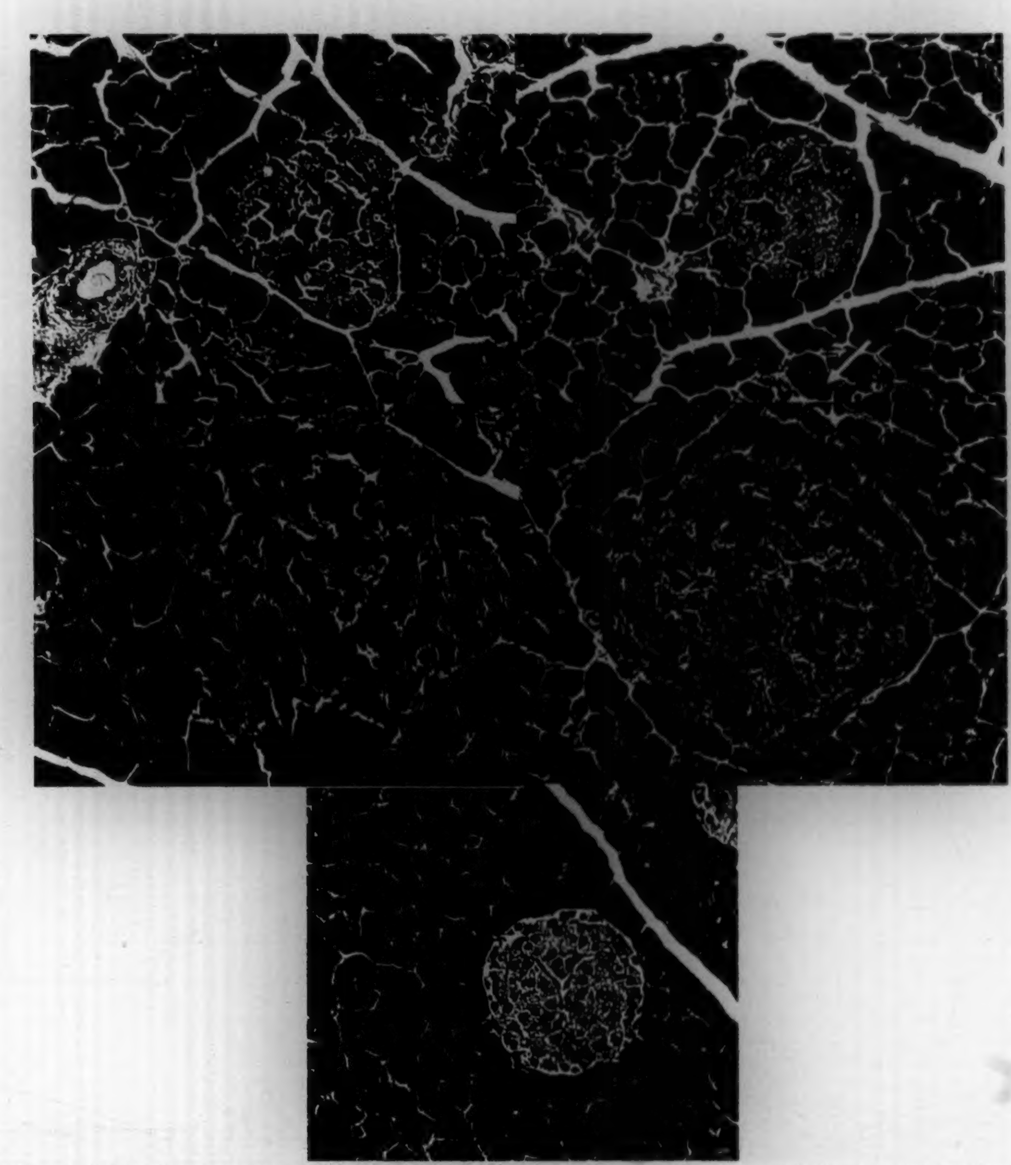


Figure 6-15: Hematoxylin and Eosin staining of pancreatic tissue containing an islet after 24 hours of preservation (magnification:*400); A: UW; B: UWO; C: UWG; D: UWGO F: Fresh;

cells are usually paler than the acinar cells. Preserving pancreas in UW solution for 24 hrs showed that almost all of central cells within the islet became heterochromatic (Figure 6-15 A). Presumably the darker-staining nuclei are those of cells that are not transcriptionally active. The structure of cells and the capillaries has been totally destroyed and there were lots of shrinkage. Nucleoli were not seen and nucleus shape was changed. In UWG group (Figure 6-15B) the situation is worse and most of the β-cells within the islet became heterochromatic. The structure of the capillaries has been destroyed and shrinkage was seen. Nucleoli were not seen and nucleus shape was changed. UW oxygenation with continuous bubbling of oxygen and supplementation with glucose showed that most of the cells within the islet remained euchromatic (Figure 6-15 C&D). The structure of the capillaries remained intact and less shrinkage was seen intra-islet. Nucleoli were seen in some of the β-cells and nucleus shape was unchanged in many of the islets.

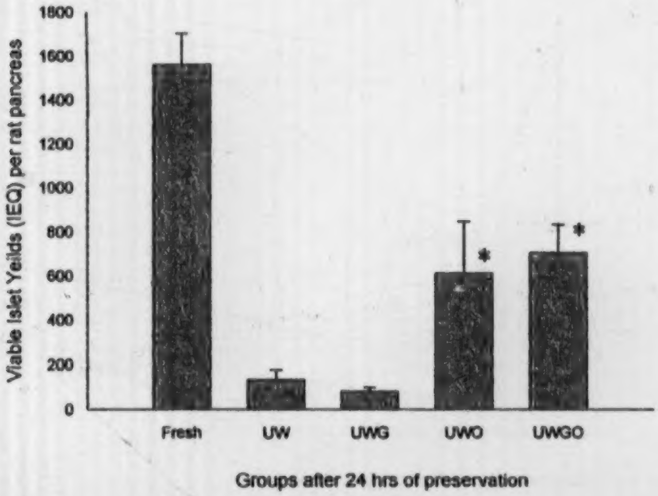


Figure 6-16: Post-purification viable islet yield after 24 hours of pancreas preservation in different groups. UW: University of Wisconsin solution, UWG: UW supplemented with 5mM glucose, UWO: UW with continuous bubbling of oxygen, UWGO: UW supplemented with 5mM glucose and continuous bubbling of oxygen. * $p<0.001$ as compared to UW and UWG groups

Islet isolation outcome

Post-purification islet yield was significantly higher in UWGO (706±129 IEQ/pancreas) and UWO (613±235 IEQ/pancreas) group as compared to UW (131±44 IEQ/pancreas, $p<0.001$) and UWG groups (79±17 IEQ/pancreas, $p<0.001$) at 24 hours of pancreas preservation (Figure 6-16). Stimulation index of isolated islets was significantly higher in UWGO group (9.00±0.67) as compared to UWG groups (6.35±1.11, $p<0.001$) at 24 hours of pancreas preservation; but not as compared to other groups (Figure 6-17).

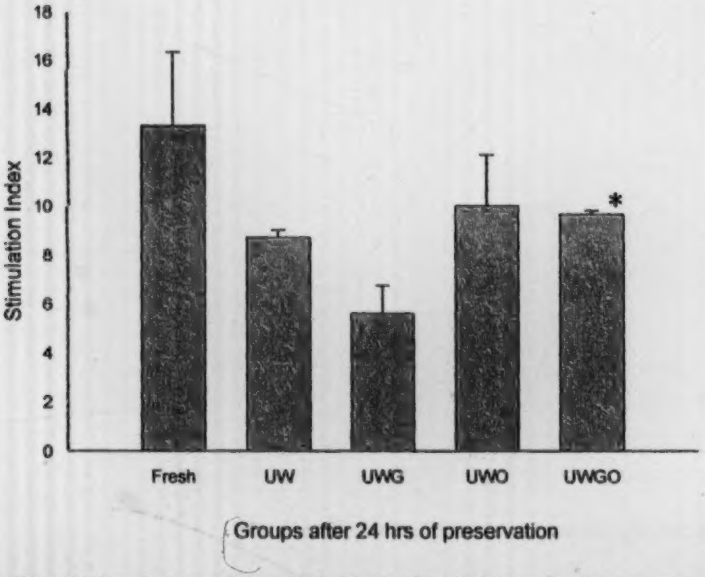


Figure 6-17: Stimulation index of isolated islets after 24 hours of pancreas preservation in different groups. UW: University of Wisconsin solution, UWG: UW supplemented with 5mM glucose, UWO: UW with continuous bubbling of oxygen, UWGO: UW supplemented with 5mM glucose and continuous bubbling of oxygen. * $p<0.001$ as compared to UW and UWG groups

Discussion

The present study found that the higher dissolved oxygen level in UW solution with continuous bubbling of oxygen could improve energy profile in pancreatic tissue. Hellerstrom found that glucose may stimulate oxygen consumption in mouse pancreatic islets [16], however glucose supplement in UW solution could not improve energy profile more than oxygenation alone. Oxygenation switches the anaerobic metabolism to aerobic one effectively, while glucose shifts it to anaerobic pathway. Recently, investigators have demonstrated that ischemia and hypoxia are associated with an influx of Na^+ into cells [17]. This influx of Na^+ is postulated to occur through activation of the Na^+-H^+ exchanger, which is stimulated by the switch from aerobic to anaerobic metabolism that occurs during hypoxia [18] or hyperglycemia. Activation of the Na^+-H^+ exchanger results in intracellular alkalinization [19]. Glucose probably activates the Na^+-H^+ exchanger that in both glucose-supplemented groups solution's pH is less than the non-supplemented ones.

Providing oxygen to a tissue leads to the production of free radicals that occurs via the hypoxanthine-xanthine oxidase reaction and can contribute to cell injury by participating in either protein peroxidation, direct DNA damage, or lipid peroxidation [20].The data showed that there was no significant lipid peroxidation by tissue oxygenation, however, the GSH values decreased significantly with bubbling of oxygen that reveals existence of some levels of free radicals. Glucose supplement could reverse that in the present study by facilitating the conversion of oxidised glutathione, which is reduced back to glutathione by the NADPH-dependent enzyme glutathione reductase (GR).

Glucose is known to dose-dependently increase the percentage of β cells in active biosynthesis and the percentage that survives during culture. It is now demonstrated that the glucose-induced survival of β cells cultured for 1 wk results from a dose-dependent reduction in the percentage of β cells dying in apoptosis (49% at 3 mM glucose, 40% at 6 mM, 9% at 10 mM). Thus, intercellular differences in glucose sensitivity appear responsible for the heterogeneity in β cell sensitivity to apoptotic conditions. These data indicate that glucose promotes survival of β cells by activating synthesis of proteins which suppress apoptosis [21]. A proportion of β cells is already biosynthetically active at low (3 mM) glucose, while the other cells become activated at higher glucose concentrations up to 10 mM. The dose-dependent recruitment of β cells into biosynthetic activity is attributed to an intercellular heterogeneity in the metabolic threshold for glucose metabolism. Prolonged exposure to 10 mM glucose maintains the majority of β cells in an activated state; in this condition, the rates of cell death and of apoptosis are low. Culture at lower glucose concentration results in lower percentages of activated β cells (10 mM > 6 mM > 3 mM) [22], and increased percentages of dying cells [10]. Since a decrease in temperature from 37°C to 4°C will decrease the metabolism by 12 fold which result in decrease of oxygen consumption by tissue [23], 5mM glucose concentration was considered optimum for the present study.

It's reported that medium oxygen-glucose deprivation induced a rapid (<12 h) mixture of apoptosis and necrosis, followed by mainly secondary necrosis [24]. Hypoglycemia/hypoxia Inducible Mitochondrial Protein (HIMP1) is expressed predominantly in α, not β, cells within the pancreas. This is perhaps why they are more sensitive to hypoglycemia and hypoxia than the other cells. Low (2.5 mM) glucose substantially increases its levels, whereas high (25 mM) glucose decreases them. HIMP1 proteins can increase β-cell survival under the stress of either hypoxic or hypoglycemic conditions [25]. The addition of glucose may enhance myocardial preservation [26]. It is suggested that the addition of 10 mmol/L glucose to UW solution is associated with enhanced recovery after prolonged hypothermic storage [27]. Glucose-induced ß-cell proliferation was also observed [28]. Hypoxia has been shown to decrease the mitochondrial potential leading to the release of cytochrome C. The released cytochrome C binds with the Apaf1/caspase 9 complex to initiate apoptosis [29]. In this study, apoptosis observed in pancreatic tissue was mitochondria-dependent, mediated by change in caspase 9 initiated by both hypoxia and hypoglycemia and mitochondria-independent mediated by change in caspase 8 initiated by hypoglycemia. There are reports showing that increased glucose concentration by itself induces apoptosis in human pancreatic ß-cells. The mechanism underlying glucose-induced ß-cell death involves the upregulation of Fas receptors, which can interact with the constitutively expressed FasL on neighboring ß-cells. Fas-FasL interaction leads to cleavage of procaspase-8 to caspase-8. Activated caspase-8, the most upstream caspase in the Fas apoptotic pathway, promotes caspase-3 activation and DNA fragmentation [30]. This study found that 5mM glucose decreases caspase 8 and suppress the extrinsic pathway of apoptosis. In contrast to that seen in human islets, an increase in glucose concentration to 11 mmol/l in rat islets promotes ß-cell survival [31,32]. When glucose concentrations were further increased, glucose proved to be pro- or anti-apoptotic, depending on culture conditions. The difference in glucose sensitivity between human and rat islets can be explained by the mechanism of glucose-induced ß-cell apoptosis. Human islets constitutively express FasL [33], whereas islets from 2- to 3-month-old rats—the age at which rats are usually investigated—do not express FasL [34]. Elevated glucose concentrations induced Fas expression in almost all ß-cells; however, apoptosis was observed in only few cells. An interesting feature of the glucose-induced apoptosis was the appearance of fragmented nuclei doublets, suggestive of postmitotic apoptosis. Therefore, susceptibility to apoptosis via Fas activation may be increased in proliferating cells. Because glucose also induces ß-cell proliferation, a relationship between induction of proliferation and apoptosis seems plausible. In line with this suggestion, exposure of ß-cells to elevated glucose concentrations induced a short-lasting increase in proliferation accompanied by long-lasting ß-cell apoptosis.

This study found that glucose supplement can delay necrosis to 12 hours after preservation as compared to bubbling of oxygen alone. Taken together, oxygenation of pancreas through bubbling plus glucose supplement of UW solution shows superior results in islet yield, function, pancreatic tissue energy profile, apoptosis, necrosis, and intracellular antioxidant capacity as compared to current pancreas preservation method.

Bibliography

[1] Hoorens A, Van de Casteele M, Klöppel G, Pipeleers D. Glucose promotes survival of rat pancreatic beta cells by activating synthesis of proteins which suppress a constitutive apoptotic program. J Clin Invest. 1996 Oct 1;98(7):1568-74.

[2] Van de Casteele M, Kefas BA, Cai Y, Heimberg H, Scott DK, Henquin JC, Pipeleers D, Jonas JC. Prolonged culture in low glucose induces apoptosis of rat pancreatic beta-cells through induction of c-myc. Biochem Biophys Res Commun. 2003 Dec 26;312(4):937-44.

[3] Moritz W, Meier F, Stroka DM, Giuliani M, Kugelmeier P, Nett PC, Lehmann R, Candinas D, Gassmann M, Weber M. Apoptosis in hypoxic human pancreatic islets correlates with HIF-1alpha expression. FASEB J. 2002 May;16(7):745-7.

[4] Wallace DC. Mitochondrial diseases in man and mouse. Science. 1999 Mar 5;283(5407):1482-8.

[5] Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell. 2004 Jan 23;116(2):205-19.

[6] Wollheim CB, Maechler P. Beta-cell mitochondria and insulin secretion: messenger role of nucleotides and metabolites. Diabetes. 2002 Feb;51 Suppl 1:S37-42.

[7] Maechler P, Wollheim CB. Mitochondrial function in normal and diabetic beta-cells. Nature. 2001 Dec 13;414(6865):807-12.

[8] Henquin JC. In: Weir GC and Kahn CR, (Eds), The Joslin's Diabetes MellitusCell Biology of Insulin Secretion, Lea & Febiger, Philadelphia, 1994, pp. 56 80.

[9] Borg LA, Andersson A, Berne C, Westman J. Glucose dependent alterations of mitochondrial ultrastructure and enzyme content in mouse pancreatic islets maintained in tissue culture: a morphometrical and biochemical study. Cell Tissue Res. 1975 Oct 13;162(3):313-21.

[10] Ling Z, Hannaert JC, Pipeleers D. Effect of nutrients, hormones and serum on survival of rat islet beta cells in culture. Diabetologia. 1994 Jan;37(1):15-21.

[11] Passonneau JV, Lowry OH. Enzymatic Analysis. A Practical Guide. Totowa, NJ: Humana Press, 1993.

[12] Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J, Protein measurement with the Folin phenol reagent, J Biol Chem 1951; 193: 265-75.

[13] Fernandez-Checa JC, Kaplowitz N. The use of monochlorobimane to determine hepatic GSH levels and synthesis. Anal Biochem 1990 Nov 1;190(2):212-9.

[14] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351-358.

[15] Nicholson, D.W., et al., Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature, **376**, 37-43 (1995).

[16] Hellerstrom C. Effects of carbohydrates on the oxygen consumption of isolated pancreatic islets of mice.Endocrinology. 1967 Jul;81(1):105-12.

[17] Haigney MCP, Miyata H, Lakatta EG, Stern MD, Silverman HS. Dependence of hypoxic cellular calcium loading on Na+-Ca2+ exchange. *Circ Res.* 1992;71:547-557.

[18] Mazzoni MC, Intaglietta M, Cragoe EJ, Arfors KE. Amiloride-sensitive Na+ pathways in capillary endothelial cell swelling during hemorrhagic shock. *J Appl Physiol.* 1992;73:1467-1473.

[19] Frelin C, Vigne P, Ladoux A, Lazdunski M. The regulation of the intracellular pH in cells from vertebrates. *Eur J Biochem.* 1988;174:3-14

[20] Boggi U, Vistoli F, Del Chiaro M, Signori S, Croce C, Pietrabissa A, Berchiolli R, Marchetti P, Prato SD, Mosca F. Pancreas preservation with University of Wisconsin and Celsior solutions: a single-center, prospective, randomized pilot study. Transplantation. 2004; 77(8):1186-90.

[21] Federici M, Hribal M, Perego L, Ranalli M, Caradonna Z, Perego C, Usellini L, Nano R, Bonini P, Bertuzzi F, Marlier LN, Davalli AM, Carandente O, Pontiroli AE, Melino G, Marchetti P, Lauro R, Sesti G, Folli F. High glucose causes apoptosis in cultured human pancreatic islets of Langerhans: a potential role for regulation of specific Bcl family genes toward an apoptotic cell death program. Diabetes. 2001;50(6):1290-301.

[22] Kiekens, R., In't Veld, P.A., Mahler, T., Schuit, F., Van De Winkel, M., Pipeleers, D. (1992) Differences in glucose recognition by individual rat pancreatic B cells are associated with intercellular differences in glucose-induced biosynthetic activity. J. Clin. Invest. 89, 117-125

[23] Belzer FO, Southard JH. Principles of solid-organ preservation by cold storage. Transplantation. 1988; 45(4): 673-6.

[24] Kalda A, Eriste E, Vassiljev V, Zharkovsky A. Medium transitory oxygen-glucose deprivation induced both apoptosis and necrosis in cerebellar granule cells. Neurosci Lett. 1998 Jan 2;240(1):21-4.

[25] Wang J, Cao Y, Chen Y, Chen Y, Gardner P, Steiner DF. Pancreatic beta cells lack a low glucose and O2-inducible mitochondrial protein that augments cell survival. Proc Natl Acad Sci U S A. 2006 Jul 11;103(28):10636-41.

[26] Fremes SE, Furukawa RD, Li RK, Tumiati LC, Weisel RD, Mickle DA. In vitro assessment of the effects of glucose added to the University of Wisconsin solution on myocyte preservation. Circulation. 1992 Nov;86(5 Suppl):II289-94.

[27] Fremes SE, Guo LR, Furukawa RD, Mickle DA, Weisel RD. Cardiac storage with UW solution and glucose. Ann Thorac Surg. 1994 Nov;58(5):1368-72

[28] Bonner-Weir S, Deery D, Leahy JL, Weir GC: Compensatory growth of pancreatic ß-cells in adult rats after short-term glucose infusion. Diabetes 38:49 53, 1989

[29] Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997; 91: 479 489.

[30] Stennicke HR, Salvesen GS: Caspases-controlling intracellular signals by protease zymogen activation. Biochim Biophys Acta 1477:299 306, 2000

[31] Donath MY, Gross DJ, Cerasi E, Kaiser N: Hyperglycemia-induced ß-cell apoptosis in pancreatic islets of Psammomys obesus during development of diabetes. Diabetes 48:738 744, 1999

---

[32] Hoorens A, Van, DC, Kloppel G, Pipeleers D: Glucose promotes survival of rat pancreatic beta cells by activating synthesis of proteins which suppress a constitutive apoptotic program. J Clin Invest 98:1568 1574, 1996

[33] Loweth AC, Williams GT, James RF, Scarpello JH, Morgan NG: Human islets of Langerhans express Fas ligand and undergo apoptosis in response to interleukin-1ß and Fas ligation. Diabetes 47:727 732, 1998

[34] Hanke J. Apoptosis and occurrence of Bcl-2, Bak, Bax, Fas and FasL in the developing and adult rat endocrine pancreas.Anat Embryol (Berl). 2000 Oct;202(4):303-12.

Chapter Seven

Conclusions

Dissertation Findings and Implications

Intra-ductal injection strategy, in chapter 3 of this thesis, showed no significant improvement in oxygenating the pancreas with the studied oxygen carriers. PFC improved energy profile as compared to the other groups after 12 hours of preservation but the level of improvement did not reach to that of fresh biopsies. There are reports available showing that intraductal injection of nutrients improves the islet isolation outcomes. Dr. Hering et al. showed that the islets isolated with their ductal preservation method (intraductal injection of UW solution alone in Lewis rat pancreas) functioned well, both in vitro (after overnight culture) and in vivo after transplantation. [1]. Avila et al. showed that intraductal glutamine administration at the time of enzyme perfusion considerably improves the islet yield, viability, and augments endogenous glutathione levels in pancreata procured after a clinically relevant period of ischemia [2].

In chapter 4 of this thesis we found that continuous bubbling of oxygen alone in preservation solution (even in a simple solution like HBSS) can improve the pancreatic tissue energy profile. It improved energy profile significantly at 4 hours of pancreas preservation and afterwards. The energy profile reached surprisingly to those of fresh values after 24 hours. Measuring of tissue oxygen tension (PO2) of the ischemically damaged pancreas graft directly during preservation either by the two-layer (UW/PFC) method or simple storage in UW with oxygen bubbling at 20°C has shown that the pancreas is not oxygenated during preservation in UW with continuous oxygen bubbling [3]. This is the first study investigating the effects of bubbling of oxygen on pancreas in hypothermic preservation condition (4°C). There is a report demonstrates that without oxygen bubbling, the two-layer method is not effective for long time small bowel preservation [4].

There are several preservation solutions that have been designed to address biological and physiological requirements for survival of pancreas in a low temperature environment. Despite major differences in constituents of all existing preservation solutions, they have broadly similar effects on islets and it is well accepted that the cold ischemia time should be kept as short as possible. During the last 3 decades Perfluorochemical-based preservation (TLM) shown to be the only method available that supplies oxygen to the pancreas during preservation [5]. However, compelling evidence that PFC alone or in combination with UW overcomes the problems associated with human pancreas preservation prior to islet isolation is lacking and difficult to obtain. Lakey et al. have previously reported some preliminary data concerning increased human islet recovery using two-layer method compared to simple UW cold storage and improved function from transplanted islets isolated through this technique [6]. However, in their recently published article they found that transplanted mass and functional viability of islet isolated from TLM-preserved pancreas were similar to those from UW-preserved pancreas. Their findings bring into question the true merit of routine use of TLM prior to islet isolation [7].

In a recently published paper, pO_2 was measured using fiber optic sensors in the core of porcine pancreatic tissue preserved with TLM in media saturated with 100% oxygen. Experimental measurements verified that pO_2 is virtually zero in the core of a 1cm-thick pancreatic piece preserved with the TLM [8]. It is believed that tissue oxygenation limitations with TLM are due to diffusion limitations of oxygen within tissue. Based on the Fick's diffusion law, Avgoustiniatos et al. have predicted fraction of rat and human pancreas volume that is oxygenated by diffusion during preservation with the TLM at 4°C. This fraction is 100% for the 1- to 2-mm thick head region of a rat pancreas, close to 60% for the 4-mm thick splenic region of a rat pancreas, and less than 20% for a cylindrical 25-mm diameter pancreas (simulating a human organ) [9]. For the studies presented in this thesis, rat model was selected to eliminate the confounding factors of diffusion with human pancreas; however the limitation of oxygenation with TLM still remained as an obstacle. Brandhorst et al. reported that omitting the University of Wisconsin (UW)-layer accelerate oxygen release into the ambient atmosphere by measurement of oxygen tension in PFC pre-charged for One-Layer-Method as compared to that for TLM. Years later, the same group showed that there is no difference between pre-oxygenated TLM (static TLM) and the original TLM with continuous oxygen supply [10]. This thesis showed that dissolved oxygen level in UW solution with continuous bubbling of oxygen is higher as compared to pre-oxygenated TLM. In spite of the huge amount of dissolved oxygen in the PFC layer, it was released partially to the UW solution in TLM group.

The continuous bubbling of oxygen improves energy profile of pancreatic tissue during 24 hours of preservation comparable to the fresh values and switches the anaerobic metabolism to aerobic one effectively. The data shows that TLM also improves the pancreatic tissue energy profile but after 12 hours of preservation and the values never reach to the fresh values and it cannot switch the anaerobic metabolism to aerobic one effectively. Hellerstrom found that glucose may stimulate oxygen consumption in mouse pancreatic islets [11], however glucose supplement in UW could not improve energy profile more than oxygenation alone. It also found that oxygenation switches the anaerobic metabolism to aerobic one effectively, while glucose shifts it to anaerobic pathway.

It's reported that medium oxygen-glucose deprivation induced a rapid (<12 h) mixture of apoptosis and necrosis, followed by mainly secondary necrosis [12]. HIMP1 (Hypoglycemia/hypoxia Inducible Mitochondrial Protein) is expressed predominantly in α, not β, cells within the pancreas. This is perhaps why they are more sensitive to hypoglycemia and hypoxia than the other cells. Continuous bubbling of oxygen alone is not successful in preventing hypothermia induced necrosis. However glucose supplement could delay necrosis to 12 hours after preservation as compared to bubbling of oxygen alone.

Providing oxygen to a tissue leads to the production of free radicals that occurs via the hypoxanthine-xanthine oxidase reaction and can contribute to cell injury by participating in either protein peroxidation, direct DNA damage, or lipid peroxidation [13]. The data showed that there was no significant lipid peroxidation by tissue oxygenation, however, the GSH values decreased significantly with bubbling of oxygen that reveals existence of some levels of free radicals. Glucose supplement could reverse that in this study by facilitating the conversion of oxidised glutathione which is reduced back to glutathione by the NADPH-dependent enzyme glutathione reductase (GR).

Isolated islets before transplantation sustained apoptosis at a high rate when the pancreas was preserved before islet isolation by conventional cold storage in UW compared with no preservation, but that apoptosis of isolated islets decreased significantly by using TLM as a preservation technique, but not to the degree as with no preservation. Hypoxia has been shown to decrease the mitochondrial potential leading to the release of cytochrome C. The released cytochrome C binds with the Apaf1/caspase 9 complex to initiate apoptosis [14]. An observation indicates that preservation of pancreata in PFC reduces organ hypoxia during cold preservation, resulting in preservation of the mitochondrial redox potential and reduction in apoptosis mediated through the mitochondrial pathway [15]. Our data show that continuous bubbling of oxygen prevents apoptosis in a more effective and consistent way during 12 hours of preservations as compared to TLM. Apoptosis observed in pancreatic tissue was mainly mitochondria-dependent, partly mediated by change in caspase 9 initiated by hypoxia. We did not observe any extrinsic pathway (caspase 8) dependent change by hypoxia alone. However, glucose supplement showed significant decrease in caspase 8 values. There are reports showing that increased glucose concentration by itself induces apoptosis in human pancreatic ß-cells. The mechanism underlying glucose-induced ß-cell death involves the upregulation of Fas receptors, which can interact with the constitutively expressed FasL on neighboring ß-cells. Fas-FasL interaction leads to cleavage of procaspase-8 to caspase-8. Activated caspase-8, the most upstream caspase in the Fas apoptotic pathway, promotes caspase-3 activation and DNA fragmentation [16].

In the present dissertation, Intraductul oxygenation of pancreas during hypothermic organ preservation could not improve pancreatic tissue energy profile, so could not be considered as a suitable way to improve islet survival during preservation time. Continuous bubbling of oxygen during hypothermic organ preservation improved pancreatic tissue energy profile and could be an alternative method for pancreas preservation, however to extend these data to human diffusion of oxygen throu the solution and the tissue as confounding factor has to be further studied. Apoptosis observed in pancreatic tissue during hypothermic preservation was prevented by providing oxygen to the tissue. It increased ATP values through activation of mitochondria,and prevented DNA fragmentation mediated by change in caspase 9 initiated by both hypoxia and hypoglycemia and mitochondria-independent mediated by change in caspase 8 initiated by hypoglycemia and probably necrosis and the release of cytokines from the other cells. Glucose supplement improved pancreatic tissue antioxidant capacity during continuous oxygenation of hypothermic preserved pancreas. Big changes of DNA fragmentation, small changes in caspases, and increase in mitochondrial function leads to thinking to another possible pathway, so called caspase-independent apoptosis (AIF, and EndoG) which needs to be further studied.

Future Directions

Taken together, oxygenation of pancreas through bubbling plus glucose supplement of UW solution shows superior results in islet yield, function, pancreatic tissue energy profile, apoptosis, necrosis, and intracellular antioxidant capacity, islet yield and function as compared to current pancreas preservation method. Although the activation of caspase is most likely a predominant mechanism inducing apoptosis, apoptosis could be mediated by mechanisms that do not involve caspases that need to get clarified. This pathway, termed caspase-independent apoptosis, is characterized by a large scale DNA fragmentation with an early chromatin condensation pattern. Caspase-independent factors, such as apoptosis inducing factor (AIF), endonuclease G (Endo G) and High temperature requirement protein A2 (HtrA2/Omi), might be involved in inducing apoptosis without mediation of caspases in pancreas hypothermic preservation.

This preservation method has to be tested in large animals and human pancreas since its positive effects could have a huge impact on the field of pancreas preservation. While multiple-donor transplants continue to be the norm, single-donor to single-recipient transplantation may become a reality as this method of pancreas preservation works. It may be even possible to treat multiple recipients from a single pancreas. Live donation of a segmental-graft specifically for islet transplantation (perhaps by laparoscopic and hand-assisted removal) is an attractive avenue that could be real by using this method.

Bibliography

[1] Sawada T, Matsumoto I, Nakano M, Kirchhof N, Sutherland DE, Hering BJ. Improved islet yield and function with ductal injection of University of Wisconsin solution before pancreas preservation. Transplantation. 2003 Jun 27;75(12):1965-9.

[2] Avila JG, Tsujimura T, Oberholzer J, Churchill T, Salehi P, Shapiro AM, Lakey JR. Improvement of pancreatic islet isolation outcomes using glutamine perfusion during isolation procedure. Cell Transplant. 2003;12(8):877-81.

[3] Matsumoto S, Kuroda Y, Hamano M, Kim Y, Suzuki Y, Ku Y, Saitoh Y. Direct evidence of pancreatic tissue oxygenation during preservation by the two-layer method. Transplantation. 1996 Dec 15;62(11):1667-70.

[4] Kuroda Y, Sakai T, Suzuki Y, Tanioka Y, Matsumoto S, Kim Y, Fujita H, Hamano M, Hasegawa Y, Ku Y, Saitoh Y. Small bowel preservation using a cavitary two-layer (University of Wisconsin solution/perfluorochemical) cold storage method. Transplantation. 1996 Feb 15;61(3):370-3.

[5] Lakey JR, Tsujimura T, Shapiro AM, Kuroda Y. Preservation of the human pancreas before islet isolation using a two-layer (UW solution-perfluorochemical) cold storage method. Transplantation. 2002; 74(12):1809-11.

[6] Lakey JR, Tsujimura T, Shapiro AM, Kuroda Y. Preservation of the human pancreas before islet isolation using a two-layer (UW solution-perfluorochemical) cold storage method. Transplantation 2002; 74: 1809-11.

[7] Kin T, Mirbolooki M, Salehi P, Tsukada M, O'Gorman D, Imes S, Ryan EA, Shapiro AM, Lakey JRT. Islet isolation and transplantation outcomes of pancreas preserved with University of Wisconsin solution versus two-layer method using pre-oxygenated perfluorocarbon. Transplantation . 2006: 82(10):

[8] Papas KK, Hering BJ, Gunther L, Rappel MJ, Colton CK, Avgoustiniatos ES. Pancreas oxygenation is limited during preservation with the two-layer method. Transplant Proc. 2005; 37(8):3501-4.

[9] Avgoustiniatos ES, Hering BJ, Papas KK. The rat pancreas is not an appropriate model for testing the preservation of the human pancreas with the two-layer method Transplantation. 2006 ;81(10):1471-2

[10] Matsumoto S, Rigley TH, Qualley SA, Kuroda Y, Reems JA, Stevens RB. Efficacy of the oxygen-charged static two-layer method for short-term pancreas preservation and islet isolation from nonhuman primate and human pancreata. Cell Transplant. 2002;11(8):769-77.

[11] Hellerstrom C. Effects of carbohydrates on the oxygen consumption of isolated pancreatic islets of mice.Endocrinology. 1967 Jul;81(1):105-12.

[12] Kalda A, Eriste E, Vassiljev V, Zharkovsky A. Medium transitory oxygen-glucose deprivation induced both apoptosis and necrosis in cerebellar granule cells. Neurosci Lett. 1998 Jan 2;240(1):21-4.

[13] Boggi U, Vistoli F, Del Chiaro M, Signori S, Croce C, Pietrabissa A, Berchiolli R, Marchetti P, Prato SD, Mosca F. Pancreas preservation with University of Wisconsin and Celsior solutions: a single-center, prospective, randomized pilot study. Transplantation. 2004; 77(8):1186-90.

---

[14] Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997; 91: 479 489

[15] Ramachandran S, Desai NM, Goers TA, Benshoff N, Olack B, Shenoy S, Jendrisak MD, Chapman WC, Mohanakumar T. Improved islet yields from pancreas preserved in perflurocarbon is via inhibition of apoptosis mediated by mitochondrial pathway. Am J Transplant. 2006 Jul;6(7):1696-703.

[16] Stennicke HR, Salvesen GS: Caspases-controlling intracellular signals by protease zymogen activation. Biochim Biophys Acta 1477:299 306, 2000